Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 107 JULY 1961 No. 3

SECTION MEETINGS

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| CLEVELAND | |
| Lakeside Hospital | May 15, 1961 |
| DISTRICT OF COLUMBIA | |
| Naval Medical Center | June 1, 1961 |
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| University of Illinois | May 9, 1961 |
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| State University of Iowa | May 4, 1961 |

## Action of Choline on Lipid Phosphorylation in the Kidney, Heart and Aorta.* (26657)

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Earlier studies with the aid of radioactive phosphorus showed that administration of a single dose of choline stimulated the turnover of liver phospholipids in animals maintained on low protein-high fat diet(1) or low protein-low fat diet(2). These low protein diets produced a choline deficiency involving the liver, kidney(3) and aorta(4,5). The hemorrhagic kidney lesion was observed only in choline deficient weanling rats(3) and a single dose of choline stimulated $P^{32}$ uptake in this tissue(6). In the present investigation an attempt has been made to study the action of single dose of choline on formation of phospholipids in the heart, aorta and kidney of rats maintained on high fat-low protein diets and in acute choline deficiency by supplementation of the diet with 1% guanidoacetic acid, methyl group acceptor.

*Methods.* Male albino rats of Sprague-Dawley strain, weighing 100-150 g were divided into 2 series. The animals in Series I were maintained on a low protein-high fat (7) diet containing vitamin-free casein 5 parts, dextrin 28, sucrose 29, crisco 30, cod liver oil 2, salt mixture 4, Ruffex 2 plus the following vitamin mixture in mg per kilo of ration; dextrin 9639, thiamin 8, riboflavin 8, pyridoxine 8, nicotinic acid 241, calcium pantothenate 32, inositol 32, paraaminoben-

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* Supported in part by grant from U. S. Atomic Energy Comm.

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zoic acid 32. This purified diet containing 5% vitamin free casein (Nutritional Biochemicals Corp.) would represent approximately 0.16 g of methionine and 0.015 $\mu$g of Vit. $B_{12}$. In Series II rats were maintained on similar diet supplemented with 1% guanidoacetic acid for 2 weeks. Animals were fed *ad libitum*. In each series, 4 to 6 rats were housed in individual cages, with raised screen bottoms.

At the end of the dietary regime, the rats were stomach-tubed with a single dose of choline (100 mg in 1 ml of water, control animals received water). One minute later all animals were injected intraperitoneally with 1 ml of physiological saline containing 100 $\mu$c as $NaH_2P^{32}O_4$. Six hours later the animals were killed by decapitation and kidney, heart and aorta were removed and acid-soluble phosphorus and lipid phosphorus fractionated. The acid-soluble phosphorus was removed by extracting with 10% trichloroacetic acid (TCA) containing 0.4 M $MgCl_2$(8) and radioactivity(9) and phosphorus(10) were determined. The TCA residue was covered with alcohol, and the lipids extracted with ethanol in Soxhlet extractor for 8 hours, purified with chloroform (9). On aliquots of the chloroform solution radioactivity(9) and phosphorus were determined(10). Radioactivity was determined with an ultra-thin window, gas flow geiger tube (Tracerlab TGC-14). From these results and the radioactivity measurements, the relative specific activities of the phospholipids were calculated. The relative specific activity is the ratio of the specific activities of the phospholipid P to that of the acid soluble P. The specific activity is defined as counts per minute per aliquot (% of dose injected)/mg of phosphorus per aliquot.

A control group of rats were injected with $P^{32}$ and sacrificed at 3, 6, and 10 hours later to determine the time uptake of the isotope by the liver, heart, kidney and aorta. The acid-soluble and phospholipid P were extracted and radioactivity(9) and phosphorus (10) were determined on each fraction.

*Results.* The time course of incorporation of $NaH_2P^{32}O_4$ into liver, kidney, heart and aorta phospholipids is shown in Table I. It

TABLE I. Time Course of Incorporation of $NaH_2P^{32}O_4$ into Liver, Kidney, Heart and Aorta Phospholipids.

| Time after adm. $P^{32}$, hr | No. of rats | Liver | | Kidney | | Heart | | Aorta | |
|---|---|---|---|---|---|---|---|---|---|
| | | Spec. activity | Rel. spec. activity | Spec. activity | Rel. spec. activity | Spec. activity | Rel. spec. activity | Spec. activity | Rel. spec. activity |
| 3 | 3 | 11.85 ± 1.9 | 6.22 ± .9 | 10.07 ± 2.4 | 10.7 ± .3 | 2.19 ± .1 | 2.84 ± .08 | 4.2 ± .9 | 6.58 ± .8 |
| 6 | 4 | 32.80 ± 1.2 | 19.00 ± 1.3 | 17.30 ± 1.4 | 20.5 ± .9 | 3.28 ± .1 | 3.55 ± .08 | 4.73 ± .3 | 9.30 ± .7 |
| 10 | 4 | 25.6 ± 1.0 | 24.20 ± 2.3 | 16.40 ± .7 | 24.6 ± 2.2 | 4.20 ± .1 | 6.42 ± .59 | 5.33 ± .8 | 14.60 ± 2.5 |

Figures preceded by ± sign indicate stand. dev.

is apparent that 6 hour time interval after administration of $NaH_2P^{32}O_4$ corresponds to the ascending part of the specific activity time curve where synthesis of phospholipid in the liver, kidney, heart and aorta occurs to a degree greater than their breakdown. The relative specific activity(11) expresses the relationship between isotopic concentration in a compound and in its precursor(12). Under the conditions of our experiments an increase in formation of phospholipids may be reasonably assumed when higher values for relative specific activities are found. Various factors, such as difference in the role of absorption and in the equilibrium between intra- and extra-cellular inorganic phosphorus with different specific activities may conceivably produce changes in the specific activity of the inorganic fraction. Comparison of the relative specific activity should provide a means of reducing their cause of error. Marinetti *et al.*(13) have demonstrated that the specific activity of the individual phospholipids of liver, heart and kidney at the 6 hour interval corresponds to the ascending part of specific activity time curve for lecithin, phosphatidylethanolamine, sphingomyelin and phosphatidylserine.

Animals were fed a 32% fat-5% casein diet to investigate the effects of a single dose of choline on lipid phosphorylation in the heart, aorta and kidney. It has previously been shown that phospholipid turnover is greater following a single dose of choline in rats maintained on 32% fat-5% casein diet than 5% fat-5% casein diet(14). The stimulating effect of lipid phosphorylation by a single dose of choline is somewhat related to the previous diet, for it does not occur in experimental animals maintained on stock diet(2). Apparently phospholipid turnover in the liver of animals maintained on a stock diet of 25% casein-5% fat diet is at a maximum value and administration of additional lipotropic agent will not further stimulate lipid phosphorylation(15).

The effect of a single dose of choline on lipid phosphorylation in the kidney, heart and aorta is shown in Table II. To evaluate the statistical significance of the results, the *t* test of significance(16) was applied to the difference between mean for control receiving water and experimental values. It is apparent that administration of a single dose of choline stimulates lipid phosphorylation in the heart and kidney.

TABLE II. Relative Specific Activity of Tissue Phospholipids Following Single Dose of Choline.

| Material admin. | No. of rats | Kidney | Heart | Aorta |
|---|---|---|---|---|
| Water | 12 | 13.7 ± 5.1 | 5.1 ±1.0 | 10.9 ±1.3 |
| Choline | 15 | 41.8 ±12.6* | 5.9 ±1.3 | 14.4 ±4.1† |
| | + 1% guanidoacetic acid in diet | | | |
| Water | 7 | 20.9 ± 2.3 | 4.5 ± .6 | 5.6 ±1.4 |
| Choline | 8 | 38.5 ±10.9* | 6.4 ±1.2* | 8.3 ±2.0† |

Test of significance was applied to difference between means of control and experimental values. Probability for chance occurrence of this difference was: * $<0.01$; † $<0.02$.

This increase in lipid phosphorylation in these tissues is probably a direct reflection of phospholipid synthesis and not uptake of plasma phospholipids. Liver is the principal if not the sole tissue concerned not only with synthesis of plasma phospholipids but also with their removal(17). When radioactive phospholipids are injected into a hepatectomized animal very little is taken up by other tissues(18). The phospholipids synthesized in the kidney are not available to the plasma in a hepatectomized animal.

This stimulation of phospholipid synthesis by a single dose of choline becomes much more exaggerated with a statistical significant increase when a choline deficiency was produced by administration in the diet of a methyl group acceptor, guanidoacetic acid. The kidneys of the animals fed the guanidoacetic acid for 2 weeks developed a hemorrhagic appearance which is seen only in dietary choline deficiency of weanling rats (3). It has been demonstrated that a single dose of choline stimulates lipid phosphorylation in the liver of rats fed a high fat-low protein(1,6) and low fat-low protein(2) and in cellular fractions of the liver, mitochondria and nuclei of rats fed a low protein-low fat diet(19). The results of our present experiments on the kidney, heart and aorta give further evidence that choline deficiency effects the phospholipids in these tissues.

*Summary.* 1. The lipid phosphorylation affects of a single dose of choline were compared in rats maintained on low protein-high fat diet or similar diet supplemented with methyl acceptor, guanidoacetic acid. 2. Administration of a single dose of choline stimulated lipid phosphorylation in the kidney, heart and aorta.

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Received February 20, 1961. P.S.E.B.M., 1961, v107.

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## Release of a Histamine-Destroying Factor During Anaphylactic Shock in Guinea Pigs.* (26658)

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Rose and Leger(1) reported that a substance capable of destroying histamine was released in rabbits during anaphylactic shock. Code and associates(2) demonstrated such a substance, probably histaminase, in the blood of white rats during anaphylactic shock. The present study was done to determine whether a similar histamine-destroying factor is released during anaphylaxis in guinea pigs.

*Methods and materials.* A group of 22 guinea pigs (700 to 1100 g) of both sexes of our mongrel laboratory strain was sensitized by 4 subcutaneous injections of crystalline egg albumin given at intervals of 24 to 48 hours. The animals were used 3 weeks to 3 months after these sensitizing injections.

During the studies of anaphylaxis, the animals were anesthetized by intraperitoneal administration of pentobarbital sodium in a dose of 3 mg per 100 g of body weight. A catheter of No. 60 polyvinyl tubing was inserted into the right atrium *via* the right jugular vein for withdrawal of samples of blood. After a control sample had been taken, 2 mg of crystalline egg albumin in 0.3 ml of an isotonic solution of sodium chloride was given *via* the catheter. Three to 5 minutes later, a specimen of blood was drawn while the animal was in shock. All samples of blood were drawn in a syringe that had

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* This investigation was supported in part by a grant from Louis W. and Maud Hill Family Foundation.

been rinsed with a solution containing 1000 U.S.P. units of heparin per milliliter. A portion or all of each sample of blood, whether obtained as a control or after shock, was extracted immediately for histamine(3) and subsequently assayed on an isolated segment of atropinized guinea-pig ileum. The flask containing one of the control samples broke during the extraction process.

Six of the 21 control specimens were divided into 2 parts; one part was immediately extracted for histamine, and the other was incubated for 1½ hours in a constant-temperature water bath at 37°C and then extracted. Seven of the 22 shock specimens were divided into 2 parts and treated similarly.

Four additional shock specimens were divided into 3 parts; one part was extracted immediately, and to another was added 0.05 ml of Tyrode's solution per milliliter of blood, after which it was incubated as above and extracted; to the third part was added 0.05 ml of aminoguanidine solution (10 mg/ml) per milliliter of blood, after which it was incubated and extracted as for the second part.

All of the final extracts were assayed on an isolated segment of atropinized guinea-pig ileum. The incubation time of 1½ hours was chosen initially for these experiments because, in studies on the rat, Code and associates(2) had found that most of the destruction of histamine was evident in that period. To check on the necessity for this incubation period, after the above studies were completed, blood specimens from 6 of the second group of 11 guinea pigs in shock were divided into 4 parts; the first part was extracted immediately, whereas the second, third and fourth parts were incubated at 37°C for ½, 1 and 1½ hours, respectively, then extracted and assayed.

Blood from 11 of the animals was drawn at varying intervals after the intra-atrial injection of the antigen, namely 15 to 30 seconds, 1 to 1½ minutes, 2 to 2½ minutes, and 4 minutes. The blood for each withdrawal period was divided into 2 parts; one was extracted immediately and the other after incubation for 1½ hours at 37°C.

TABLE I. Blood Histamine* of Sensitized Guinea Pigs before and after Shock.

| | | After shock | | |
|---|---|---|---|---|
| Animals | Before shock | Immediate extraction | Incubation† followed by extraction | Loss of histamine after incubation, % |
| 1 | .06 | .30 | .085 | 71 |
| 2 | .06 | .32 | .04 | 87 |
| 3 | .07 | .07 | .05 | 0 |
| 4 | .025 | .34 | .04 | 88 |
| 5 | .075 | .21 | .057 | 72 |
| 6 | .05 | .425 | .105 | 75 |
| 7 | .06 | .11 | .03 | 73 |
| 8 | .08 | .16 | .08 | 50 |
| 9 | — | .57 | .09 | 84 |
| 10 | .13 | .91 | .135 | 85 |
| 11 | .06 | .66 | .05 | 92 |

* Expressed as μg of histamine/ml of whole blood.

† Incubated for 1½ hr at 37°C.

*Results.* Anaphylactic shock developed in all the animals. The first indication of such shock was seen 20 to 30 seconds after intra-atrial injection of the antigen, when the animal's lips and nose began to twitch vigorously. At about 1 minute, a sudden and violent jerking forward of the head occurred. The heart rate rapidly increased. Breathing became labored and inspiration was extremely difficult. Artificial respiration was sometimes necessary. Dyspnea increased during the second minute. Tachycardia persisted. Several of the animals displayed increased peristalsis, and some lost urinary and fecal control.

With only one exception (animal 3), all shock specimens showed a 2-fold to 14-fold increase in concentration of histamine over the control specimen (Table I). All shock specimens except in animal 3 showed a loss of 50 to 92% of the histamine after 1½ hours of incubation.

The destruction of histamine was as great after ½ hour of incubation as after 1½ hours. Concentration of histamine in the control samples of blood was the same whether they were extracted immediately or after 1½ hours of incubation.

The 4 specimens of blood from animals in shock that were incubated with aminoguanidine showed no loss of histamine.

As noted previously, 22 specimens from 11 of the animals were secured at various intervals after the shocking dose was administered. Twelve of these showed an increase in histamine as compared to values in the same animal before shock; 19 specimens showed evidence of a histamine-destroying factor. Among those specimens taken 15 to 30 seconds after injection of the antigen, a 3-fold increase in histamine was noted in one animal, a 50% increase in another and no increase in 3 animals; destruction of histamine was demonstrated in 4 of these 5 specimens. For the specimens obtained 1 to 1½ minutes after injection of the antigen, only one of the 3 animals showed an increase of histamine, and 2 of the 3 showed evidence of destruction of histamine. For those specimens taken 2 to 2½ minutes after injection of the antigen, 4 of 6 guinea pigs showed a 2-fold to 14-fold increase in histamine; each of the 6 specimens showed evidence of destruction of histamine. Four minutes after injection of the antigen, 6 of 8 animals showed a 50% to 7-fold increase in histamine; 7 of the 8 showed evidence of destruction of histamine.

No specific index of degree of clinical shock was used. However, it was evident that some animals displayed a more severe degree of shock than did others. Neither the height of blood histamine present 4 minutes after injection of the antigen nor the degree of increase of this value over control values could be correlated with the estimated degree of shock observed in the animals.

*Comment and conclusions.* A state of shock can be induced by intravenous administration of the specific antigen into guinea pigs sensitized to crystalline egg albumin and anesthetized with pentobarbital sodium. The finding that histamine is released into the blood of guinea pigs during anaphylactic shock(4) has been confirmed.

It has been shown previously that aminoguanidine blocks the action of histaminase (5,6). Since aminoguanidine blocked the destruction of histamine in this study, it has been concluded that the factor in the blood responsible for destruction was diamine oxidase (histaminase). Code's group(2) arrived at a similar conclusion from their observations on rats.

The incubation period necessary to demonstrate destruction of histamine in blood from shocked guinea pigs is much less than that in rats. A period of 1½ hours in all specimens was used initially in this study because of prior experience with rats. However, shorter incubation was tried after the first part of the study was completed. Destruction was as extensive at ½ hour as at 1½ hours. The work of Arunlakshana and co-workers(7) suggests that the rate of destruction is even more rapid than this.

No histamine-destroying activity was demonstrated in blood secured before shock. However, such activity was present in shock blood regardless of the extent to which the histamine was increased. Blood drawn at varying intervals after the shocking dose showed histamine-destroying activity even when little or no increase in histamine could be demonstrated. This was especially true of the specimens secured at 15 to 30 seconds.

However, Boreus(8) found that blood histamine increased within 30 seconds after injection of the antigen. He also noted a reduction of about 50 per cent in the tissue mast cells at this time. The prompt appearance of histaminase activity at a time of massive destruction of mast cells suggests that this activity may have been released by these cells. This possibility merits further study.

The inconsistent increase of blood histamine in the first 2 minutes after injection of antigen could be caused by concomitant release of histaminase. This also may account in part for the failure to find a correlation between increase in blood histamine and estimated degree of shock in the animals.

The technical assistance of Mr. Joseph Kennedy and Miss Margaret Hurn was invaluable in this study.

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Received April 7, 1961. P.S.E.B.M., 1961, v107.

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## Influence of Graded Levels of Progesterone in Ovariectomized Rats upon Placentomata Formation Measured by Total DNA.* (26659)

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Weichert(1) was first to produce placentomata (deciduomata) reaction in ovariectomized rats. Astwood(2) and Rothchild and Meyer(3) suggested its use in assay of progesterone, whereas Evans *et al.*(4) proposed use of this reaction in assay of luteotropic hormone. Use of DNA in quantitative study of mammary gland growth(5) suggested that a quantitative assay of progesterone or luteotropin might be developed under standardized conditions comparing DNA of normal and traumatized horns of rat uteri. It was thought that studies of the various hormones which might influence growth of the placentomata should be conducted in normal animals before hypophysectomized would be used. Present study concerns influence of graded amounts of progesterone (P) on placentomata formation.

*Materials and methods.* Sprague-Dawley-Rolfsmeyer rats fed Purina Lab Chow were maintained in animal room at 78 ± 1°F until regular estrous cycles were established. Ovariectomy (OE) was performed when rats showed fully cornified smears. Immediately after operation, progesterone was given at level of 1.5 mg/day from day 1-5 during "pretrauma period"(3). Endometrium of one uterine horn of each animal was traumatized on day 6 by inserting needle through cut tubal end of uterus as far as cervix on this side. By withdrawing needle slantwise, antimesometrial aspect of endometrium was scratched 2 times throughout its entire length by sharp needle point. Immediately after operation, P was given daily at levels of 1, 2, 3, and 6 mg respectively from day 6-9, "post-trauma period"(3), whereas control rats did not receive P during this period. Rats were killed on day 10, 24 hours after last injection. Pituitaries, adrenals and uteri were removed rapidly, transferred to ice-cold vials, weighed on Roller Smith balance and put in deep freezer. Uteri were divided before weighing into control and traumatized horn and processed for DNA(6). Traumatized horns were cut in pieces, washed in alcohol before starting alcohol and ether extraction in order to obtain dry-fat-free tissue (DFFT).

*Results.* P in graded doses stimulated a graded increase in mean total DNA in the traumatized uterine horns. One mg P increased total DNA of traumatized horn markedly as compared to rats which did not receive P during post-trauma period. Six mg P did not further increase total DNA of traumatized horn as compared to 3 mg group (Table I). Decrease of DNA/mg DFFT took place in all groups. Nine of 23 rats receiving no P during post-trauma period had placentoma reactions better than group average of 7% increase of traumatized horn over control horn. A few rats did not show reactions under influence of P. Per cent increase of wet weight, DFFT and DNA of trauma-

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* Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2286. Approved by Director. This investigation supported in part by grants of Am. Cancer Soc. and U.S.P.H.S.

† Research Fellow, Dept. of Animal Physiol. and Animal Nutrition, Univ. of Goettingen, West Germany and Medical Fellow of Population Council. Present address: Univ. of Goettingen.

TABLE I. Effect of Graded Levels of Progesterone on Placentoma Formation after Traumatization of One Uterine Horn in Ovariectomized Rats.

| Treatment* | No. of rats: Total | No. of rats: Without reaction† | Body wt, g: Day 1 | Body wt, g: Day 10 | Avg wt of uteri: Wet wt/100 g horn: Control, mg | Avg wt of uteri: Wet wt/100 g horn: Trauma, mg | Avg wt of uteri: DFFT/100 g horn: Control, mg | Avg wt of uteri: DFFT/100 g horn: Trauma, mg | DNA/mg DFFT horn: Control, μg | DNA/mg DFFT horn: Trauma, μg | DNA/mg DFFT horn: Decrease, % | DNA/100 g horn: Control, mg | DNA/100 g horn: Trauma, mg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| None | 23 | 14 | 236 | 240 | 42 ± 2 | 70 ± 5 | 8.0 ±.4 | 11 ± .7 | 55 ± 2.3 | 43 ± 1.9 | 21 ± 2.2 | .42 ±.012 | .46 ±.024 |
| 1 | 30 | 4 | 226 | 232 | 52 ± 2 | 167 ± 12 | 9.6 ±.4 | 24 ± 1.6 | 49 ± 1.4 | 35 ± .9 | 27 ± 2.2 | .46 ±.014 | .84 ±.050 |
| 2 | 27 | 1 | 224 | 228 | 53 ± 3 | 189 ± 21 | 9.2 ±.6 | 27 ± 2.8 | 53 ± 1.3 | 38 ± 1 | 27 ± 2.1 | .48 ±.033 | .96 ±.072 |
| 3 | 25 | 1 | 223 | 231 | 52 ± 2 | 238 ± 32 | 9.5 ±.4 | 32 ± 3.0 | 52 ± 1.2 | 38 ± .7 | 25 ± 2.1 | .49 ±.018 | 1.20 ±.115 |
| 6 | 19 |  | 238 | 246 | 55 ± 3 | 244 ± 25 | 9.7 ±.5 | 34 ± 2.6 | 47 ± 1.4 | 33 ± 1.3 | 28 ± 3.3 | .46 ±.021 | 1.11 ±.078 |

DFFT = dry, fat-free tissue. P = progesterone.

* Pretrauma period: All rats, day 1-5, 1.5 mg P/day. Post-trauma period: Day 6-9, mg P/day.

† When traumatized horn showed less than 7% increase of total DNA as compared to control horn.

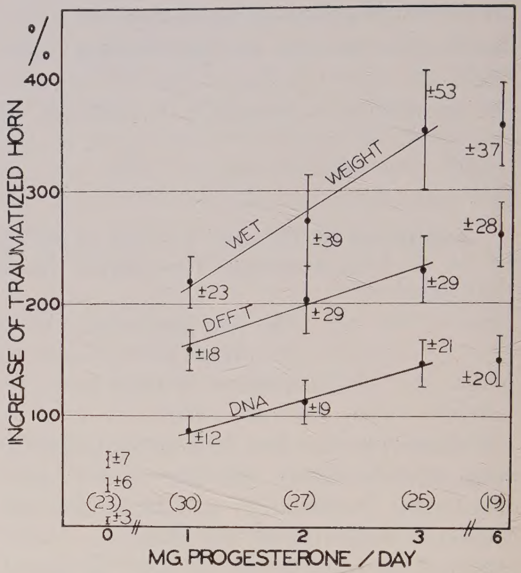

FIG. 1. Percentage weight increase of traumatized uterine horn over control horn under influence of graded levels of progesterone in ovariectomized rats. Number in parenthesis indicates number of animals.

tized horn over control horn is correlated with amount of P given during post-trauma period (Fig. 1). With exception of DFFT in 6 mg group, this level did not further improve reaction. Because of lack of uniformity of placentoma formation, standard deviations are extremely high. Body weight was not affected adversely in spite of 2 operations on day 1 and 6. Pituitary and adrenal weights remained within normal limits.

*Discussion.* Data presented is first quantitative evidence that graded amounts of P up to 3 mg/day are correlated with extent of placentoma formation in OE rats confirming previous studies based on qualitative data (2). A graded increase of wet weight, DFFT, and total DNA was observed up to 3 mg P. Similarly, the percentage weight increase of traumatized horn over control horn was shown (Fig. 1). It has been reported(7) that polyploidy occurs in growth of placentomata and that these cells cannot reproduce normally. It will be noted, however, that DNA/mg DFFT shows a decrease in every case in traumatized horns as compared to control horns. Since DNA is a nuclear constituent, one would expect DNA to increase/mg rather than decrease if it were to occur in large numbers unless cytoplasmic volume of polyploid cells increased at a rate faster than in normal cells. To extent that polyploidy does occur, total DNA/100 g would not represent true cell multiplication but rather nuclear multiplication. Since extremely great variations in placentoma reaction were observed in all groups, large number of animals will be required if this technic should be used to develop an assay method. It should be noted that in a few animals given P no reaction occurred whereas increases up to 47% in total DNA occurred in traumatized horn over control horn when no P was given during post-trauma period.

*Summary.* Sprague-Dawley-Rolfsmeyer rats were ovariectomized in estrus, then administered progesterone (P) at rate of 1.5 mg/day for 5 days (pretrauma period). Endometrium of one uterine horn of each animal was scratched throughout its entire length by sharp needle. During post-trauma period, days 6 to 9, P at levels of 1, 2, 3, and 6 mg was injected daily. On day 10 each uterus was separated into control and traumatized horn, weighed and processed for DNA determination. The traumatized horns showed a graded increase in wet weight, DFFT, and DNA/100 g B.W. over control horns when 1, 2, and 3 mg/day of P were administered during post-trauma period. Six mg/day did not increase reaction further. Considerable variation in reaction occurred in each group requiring a considerable number of animals for use as assay method.

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Received April 20, 1961. P.S.E.B.M., 1961, v107.

# Influence of Progesterone, Estradiol Benzoate, and Thyroxine in Ovariectomized Rats upon Placentomata Formation Measured by Total DNA.* (26660)

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A procedure was previously described by which a quantitative estimation of placentomata reaction might be made using DNA as an index(1). During posttrauma period increasing levels of progesterone (P) (1 to 6 mg) were injected. The DNA of traumatized horn was then compared with control horn. It was shown that 3 mg/day of P had optimal effect. In a study of growth of mammary gland of rat, it was shown that estradiol benzoate (EB) at level of 1 $\mu$g/day synergized with P at level of 3 mg/day to promote optimal growth as measured by DNA (2). The present study was conducted to determine if this level would synergize with P to promote placentomata reaction.

In the strain of rats used, the thyroxine secretion rate (TSR) has been determined. TSR varies from 0.25 $\mu$g to 2.0 $\mu$g/100 g BW with mean of about 1.0 $\mu$g. When 3.0 $\mu$g/100 g BW of L, thyroxine ($T_4$) was administered with 1 $\mu$g EB + 3 mg P, $T_4$ was with-

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* Contribution from Mo. Agr. Exp. Sta., Journal Series No. 2289. Approved by Director. This investigation supported in part by grants of Am. Cancer Soc. and U.S.P.H.S.

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TABLE I. Effect of Progesterone, Estradiol Benzoate and Thyroxine on Placentomata Formation.

| *Treatment*<br>Pretrauma period:<br>All rats, day 1-5,<br>1.5 mg P/day<br>Posttrauma period:<br>Day 6-9, mg P/day | No. of rats | Body wt, g | | Pituitary wet wt/100 g (mg) | Adrenals wet wt/100 g (mg) | Avg wt of uteri<br>Wet wt/100 g horn | | |
|---|---|---|---|---|---|---|---|---|
| | | Day 1 | Day 10 | | | Control, mg | Trauma, mg | Increase, mg |
| P, 3 mg* | 25 | 223 | 231 | 4.8 ± .08[1] | 27.1 ± 1.1 | 52 ± 2 | 238 ± 32 | 354 ± 53 |
| P, 3 mg + E.B., 1 μg | 17 | 240 | 242 | 5.8 ± .12[2] | 25.9 ± .9 | 92 ± 3 | 404 ± 36 | 341 ± 37 |
| P, 3 mg + $T_4$, 3 μg/100 g | 14 | 238 | 235 | 6.0 ± .13[3] | 27.4 ± .8 | 89 ± 4 | 396 ± 32 | 349 ± 37 |
| P, 6 mg* | 19 | 238 | 246 | 4.6 ± .12[4] | 23.4 ± .6 | 55 ± 3 | 244 ± 25 | 358 ± 37 |
| P, 6 mg + E.B., 1 μg | 13 | 243 | 246 | 6.0 ± .16[5] | 25.6 ± .6 | 85 ± 3 | 342 ± 30 | 313 ± 40 |
| P, 6 mg + E.B., 2 μg | 12 | 246 | 245 | 5.7 ± .20[6] | 25.4 ± .4 | 99 ± 8 | 341 ± 37 | 256 ± 38 |
| P, 6 mg + E.B., 2 μg + $T_4$, 3 μg/100 g | 13 | 235 | 236 | 5.8 ± .19[7] | 25.9 ± .8 | 90 ± 4 | 400 ± 18 | 355 ± 28 |

| | | Avg wt of uteri<br>DFFT/100 g horn | | | DNA/mg DFFT horn | | | DNA/100 g horn | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Control, mg | Trauma, mg | Increase, % | Control, mg | Trauma, mg | Decrease, % | Control, mg | Trauma, mg | Increase, % |
| P, 3 mg* | 25 | 9.5 ± .4[8] | 32 ± 3 [15] | 230 ± 29[22] | 52 ± 1.2[29] | 38 ± .7[36] | 25 ± 2.1[43] | .49 ±.013[50] | 1.20 ±.115[57] | 147 ± 21[64] |
| P, 3 mg + E.B., 1 μg | 17 | 16.0 ± .6[9] | 59 ± 5.4[16] | 269 ± 35[23] | 33 ± .5[30] | 25 ± .5[37] | 22 ± 2 [44] | .52 ±.020[51] | 1.48 ±.136[58] | 184 ± 24[65] |
| P, 3 mg + $T_4$, 3 μg/100 g | 14 | 16.0 ± .9[10] | 57 ± 4.7[17] | 262 ± 31[24] | 32 ± .8[31] | 25 ± .4[38] | 20 ± 2 [45] | .50 ±.027[52] | 1.43 ±.117[59] | 189 ± 23[66] |
| P, 6 mg* | 19 | 9.7 ± .5[11] | 34 ± 2.6[18] | 261 ± 28[25] | 47 ± 1.4[32] | 33 ±1.3[39] | 28 ± 3.3[46] | .46 ±.021[53] | 1.11 ±.078[60] | 150 ± 20[67] |
| P, 6 mg + E.B., 1 μg | 13 | 14.5 ± .6[12] | 49 ± 4.1[19] | 247 ± 34[26] | 32 ± .8[33] | 26 ± .5[40] | 17 ± 1.9[47] | .46 ±.022[54] | 1.28 ±.102[61] | 184 ± 25[68] |
| P, 6 mg + E.B., 2 μg | 12 | 17.0 ±1.1[13] | 51 ± 5.7[20] | 208 ± 34[27] | 30 ± .8[34] | 25 ± .7[41] | 15 ± 3.9[48] | .51 ±.042[55] | 1.28 ±.139[62] | 159 ± 28[69] |
| P, 6 mg + E.B., 2 μg + $T_4$, 3 μg/100 g | 13 | 14.6 ± .6[14] | 57 ± 2.4[21] | 302 ± 25[28] | 33 ± .8[35] | 29 ± .4[42] | 13 ± 2.0[49] | .48 ±.015[56] | 1.65 ±.093[63] | 245 ± 20[70] |

DFFT = Dry, fat-free tissue. E.B. = Estradiol benzoate. P = Progesterone. $T_4$ = Thyroxine.

* Data from Paper (1).

Student's "t" test

| | |
|---|---|
| 1–2, 3<br>4–5, 6, 7<br>8–9, 10<br>11–12, 13, 14<br>15–16, 17<br>18–21<br>29–30, 31<br>32–33, 34, 35<br>36–37, 38<br>39–40, 41<br>41–42<br>60–63 | $<.001$ |
| 18–19, 20<br>46–49<br>67–70 | $.001 < p < .005$ |
| 31–35<br>39–42<br>46–47, 48<br>69–70 | $.01 < p < .025$ |
| 27–28<br>62–63 | $.025 < p < .050$ |
| 13–14<br>43–45<br>50–51<br>57–58<br>60–61<br>64–65 | $.1 < p < .2$ |
| 20–21<br>22–23<br>25–27, 28<br>43–44<br>53–54, 55<br>57–59<br>60–62<br>64–66<br>67–68 | $.2 < p < .4$ |
| 22–24<br>53–56<br>55–56 | $.4 < p < .5$ |
| 25–26<br>48–49<br>50–52<br>67–69 | $>.5$ |

out synergistic effect on mammary gland growth but when ovarian hormone levels were doubled, $T_4$ stimulated a significant increase of 26% in total DNA of mammary glands(3). It was considered of interest to determine whether these levels of hormones would promote the placentomata reaction as well.

*Material and methods.* During the pretrauma period, rats of Sprague-Dawley-Rolfsmeyer strain were treated exactly as described previously(1). During the post-trauma period, groups of rats received 1 μg EB + 3 mg P, 3 mg P + $T_4$; 3 μg/100 g BW, 6 mg P + 1 or 2 μg EB, and finally, 6 mg P + 2 μg EB + $T_4$; 3 μg/100 g BW for 4 days and sacrificed on day 5. Uteri were divided and control and traumatized horns separated, weighed and processed for determination of DNA(4).

*Results.* Injection of 1 μg EB with 3 mg P stimulated an increase in wet and dry fat-free (DFFT) weight of both control and traumatized horns of uterus in comparison with P alone. However, the % increase in DFFT/100 g BW of traumatized *vs* control was not significant. The same is true of DNA/100 g BW. When $T_4$ was injected with 3 mg P, wet and DFFT, and DNA/100 g BW were quite similar to results observed with estrogen.

Increasing levels of EB and P were without significant effect upon any of the indices of placentomata production; however, injection of 2 μg EB + 6 mg P + 3 μg/100 g BW thyroxine stimulated a highly significant increase in all indices.

Administration of estrogen stimulated significant increases in pituitary weight but adrenal weights were not influenced.

*Discussion.* The value of DNA technic in quantitative study of hormones influencing placentomata growth has been reported. It was shown that EB added to P at levels of 1 or 2 μg increased both wet and dry, fat free tissue of the control horn as would be expected(5). It was unexpected to observe that $T_4$ had a similar effect.

EB also increased markedly wet and DFFT tissue of traumatized horns. However, % increase of traumatized horns over control horns was unchanged in comparison to P alone. The DNA/100 mg BW in control horns with addition of either EB or $T_4$ was quite similar to P alone. However, in traumatized horns EB and $T_4$ induced a slight but not significant increase in total DNA/100 g BW.

While EB 2 μg + P 6 mg was without marked effect, when $T_4$, 3 μg/100 g was added there was a significant increase in all indices of placentomata stimulation. A similar increase in mammary gland growth, as measured by total DNA, has been observed by same levels of these 3 hormones(3). Observation that 2 separate tissues (uterus and mammary glands) of reproductive system of rat have shown a significant growth response to same levels of these 3 hormones is of interest.

It was pointed out(1) that great variability in response to P was observed. It will be noted that variability in DNA/100 g BW in traumatized horns under 3 hormones was reduced. It is suggested that variability due to thyroxine secretion rate was reduced by use of optimal level of $T_4$.

*Summary.* Progesterone (P) has been shown to stimulate placentomata formation in traumatized horns of rat uteri. Influence of estradiol benzoate (EB) and thyroxine ($T_4$) as possible synergists in this reaction using DNA as an index was studied. Slight, but not significant, increases in various indices of placentomata growth were observed when EB or $T_4$ were administered separately with P. Highly significant increases were observed, however, when 2 μg EB + 6 mg P + 3 μg $T_4$/100 g BW were administered. The same level of these 3 hormones stimulated a significant increase in mammary gland growth, indicating similar response in 2 tissues of reproductive system.

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Received April 20, 1961. P.S.E.B.M., 1961, v107.

# Chronic Catheterization of Coronary Artery: Induction of ECG Pattern of Myocardial Ischemia by Intracoronary Epinephrine.*† (26661)

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The relationship between "disturbances of the mind" and angina pectoris was noted by Heberden in his original description of the disease(1); the subsequent disability of his friend, John Hunter, and the latter's death following an argument, served to focus attention on the role of emotional factors in causation of coronary artery disease. In the two centuries which have elapsed since Heberden's account, only scattered reports have been published of experimental data linking "nervous tension" and coronary disease(2). Recently, Magatsian and co-workers(3,4) have described myocardial damage produced in baboons and monkeys by long-continued psychic stress. Friedman *et al.*(5) have reported that "men exhibiting a behavior pattern associated with coronary artery disease" have a high urinary excretion rate of catechol amines. In an attempt to determine whether the alleged effect of "nervous stress" in the pathogenesis of coronary disease may be mediated by neurohumoral transmitters acting directly on the heart, a technic for chronic catheterization of the coronary artery has been developed. Such a preparation affords the opportunity to examine the acute and chronic effects of localized, intracoronary infusions of epinephrine and norepinephrine on the electrocardiogram of the unanesthetized dog.

*Methods.* Chronic catheterization of the coronary artery has been successfully performed in 5 male dogs (25-50 kg). The animals were anesthetized with pentobarbital and placed on positive pressure respiration. After thoracotomy (left fourth interspace) the pericardium was incised and the edges sutured to the chest wall, forming a cradle for the heart. The left anterior descending coronary artery was dissected from its bed for 4 to 5 cm, umbilical tapes were passed beneath the artery (Fig. 1A), and a double purse-string suture (6-0) was placed superficially in the wall of the artery (Fig. 1B). A polyvinyl catheter (I.D.-0.38 mm and O.D.-0.76 mm) was prepared with a cuff 10 mm from the end (Fig. 1B). To stiffen and facilitate handling the soft catheter a steel wire was inserted to the tip of the tubing. A sharp, short bevel needle was used to make a hole through the arterial wall in the center of the purse-string sutures. The umbilical tapes were pulled in opposite directions to immobilize and occlude the artery momentarily, and the catheter was inserted through the hole up to the cuff. The purse-string sutures were then tied, and looped above the cuff to prevent accidental withdrawal of the catheter. After removal of the wire, the free end of the catheter was passed through the chest wall *via* a large needle. The catheter was filled with heparin (100 mg/ml), and

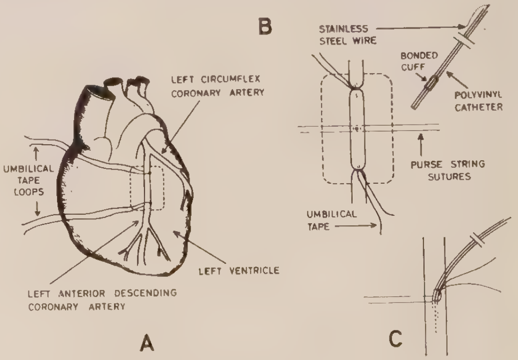

FIG. 1. Operative technic for chronic implantation of catheter into left anterior descending coronary artery.

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* Supported in part by grants from Massachusetts Heart Assn. (Greater Boston Chapter), U.S.P.H.S., and from the Eugene Higgins Trust through Harvard University.

† We wish to thank Drs. David G. Freiman, Paul M. Zoll and Ralph E. Thiers for advice and aid in these studies, Dr. Harold Upjohn for a generous supply of heparin, Smith, Kline & French Lab. for Dibenzyline used in these studies, and F. W. Smith for technical assistance.

‡ Fellow of Life Insurance Medical Research Fund.

§ Fellow of National Foundation.

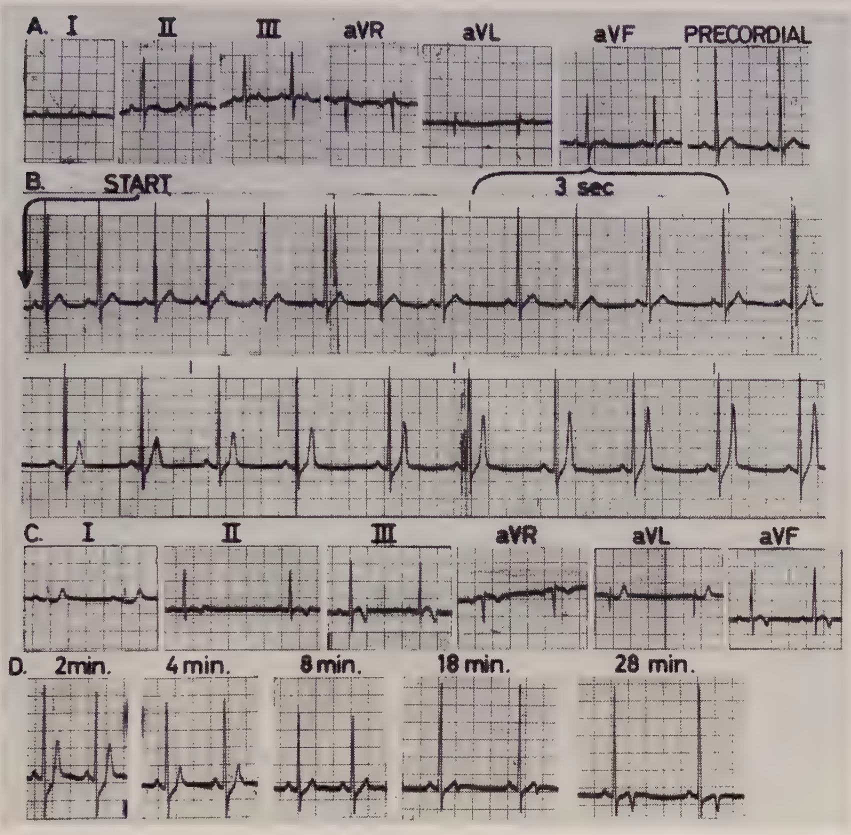

FIG. 2. Electrocardiographic changes induced by intracoronary infusion of l-epinephrine in unanesthetized dog. A. Control. B. Continuous tracing of precordial lead during infusion of 2.0 μg/min. C. Limb leads taken 2 min. later while infusion was continued. D. Precordial lead at 2, 4, 8, 18 and 28 min. after cessation of 1 hr infusion.

sealed with a stainless steel obturator. The pericardium was loosely approximated before closing the chest. In the operations which proceeded uneventfully, the animals were up and eating 8 to 12 hours postoperatively. The catheter was refilled with heparin each day. Although the electrocardiograms were normal by the tenth day, experiments were not performed during the first 3 postoperative weeks. During this period the animals were trained to lie quietly on the right side while electrocardiograms were taken. All infusions were administered through the coronary catheter at the rate of 0.25 ml/min, with the animals in the fasting state. Before infusion of the drugs (dissolved in Krebs-Henseleit solution), control electrocardiograms were recorded while the diluent alone was administered.

*Results.* Uniform changes in the electrocardiogram were noted in all the dogs during the intracoronary infusion of l-epinephrine hydrochloride (1.0-4.0 μg/min of the base) or l-norepinephrine bitartrate (0.4-4.0 μg/min of base), with l-norepinephrine being 3 to 4 times more potent. Within 10 seconds after onset of the infusion of l-epinephrine the amplitude of the precordial T wave began to increase, reaching a maximum height in 30-60 seconds; simultaneously the ST segment became depressed, and the R and S waves increased slightly in amplitude (Fig. 2B). Changes in the limb leads are depicted in Fig. 2C. If the infusion of the sympathomimetic amines was continued for 1-2 hours, a characteristic electrocardiographic pattern gradually developed following cessation of the infusion (Fig. 2D). There was a depression and upward coving of the ST segment, and inversion of the T wave; these abnor-

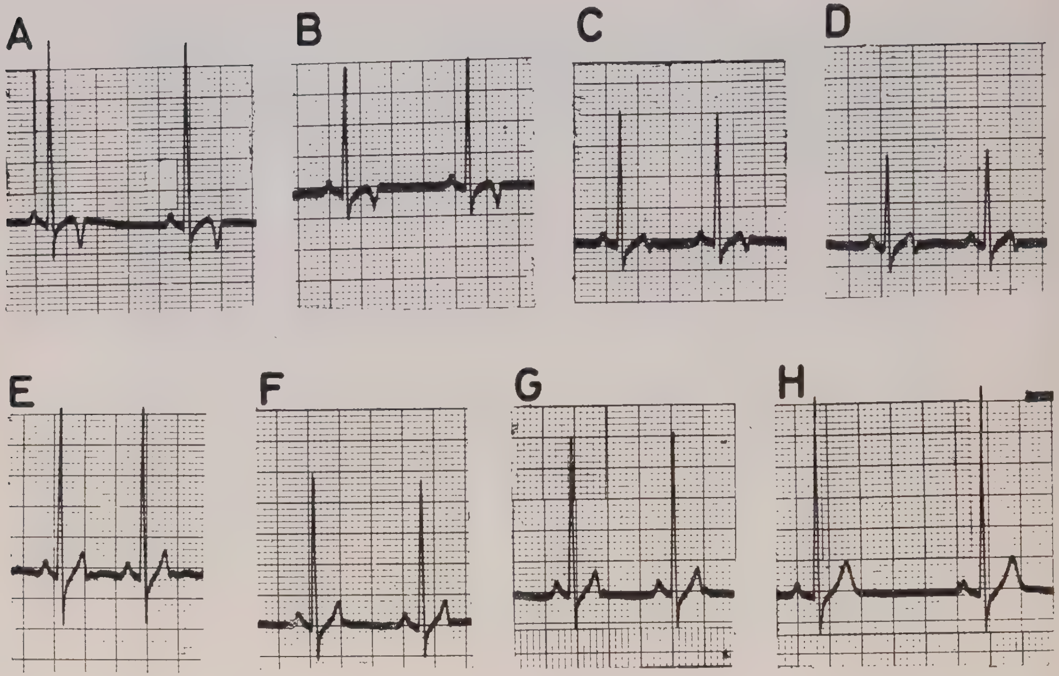

FIG. 3. Restoration of electrocardiogram (precordial) to normal by intracoronary infusion of phenoxybenzamine (100 $\mu$g/min.). A. Precordial lead 24 hr after l-epinephrine (control). B.-E. 3, 6, 16 and 30 min. after start of infusion of phenoxybenzamine. Infusion stopped at E. F.-H. 15, 30 and 120 min. post-infusion.

malities persisted for 7-10 days. The behavior of the animals did not suggest any discomfort during this period, and serum lactic dehydrogenase levels were within the normal range. One animal was sacrificed after 3 months of nearly daily infusions of epinephrine or norepinephrine. The heart was normal in appearance, and injection of the coronary artery by the Schlesinger technic(6) revealed patency of all of the coronary arteries. The intracoronary portion of the catheter was enclosed in a sleeve of endothelium open only at the catheter tip. Microscopic examination of the heart showed normal myocardium and coronary arteries.

The abnormal postinfusion electrocardiogram produced by epinephrine or norepinephrine was restored to normal temporarily by intracoronary infusions of 1) l-epinephrine (0.2-.3 $\mu$g/min), 2) l-norepinephrine (0.1 $\mu$g/min), 3) acetylcholine (25 $\mu$g/min), 4) procaine hydrochloride (0.5 mg/min) and 5) phenoxybenzamine hydrochloride (Dibenzyline 100 $\mu$g/min). Infusion of low concentrations of the sympathomimetic drugs reverted the T waves to normal, while larger doses produced high, peaked T waves and depressed ST segments as noted above. When infusion of either concentration was stopped, however, the inverted T waves reappeared within several minutes. Acetylcholine produced only a transient restoration of the normal electrocardiogram, with inversion of the T wave reappearing during the infusion. Phenoxybenzamine was slower in onset of action (Fig. 3) but the persistence of the normal ECG was longer than noted with the other drugs mentioned above.

*Discussion.* The acute electrocardiographic changes noted during intracoronary infusion of the catechol amines in the present study were probably the result of increased rate of depolarization and repolarization in the area supplied by the left anterior descending coronary artery. Hammer and Pisa (7) have, in fact, observed a late systolic bulging of the anterior wall of the left ventricle during intracoronary administration of norepinephrine; the area infused was thus repolarized and relaxing, while the rest of the left ventricular muscle was still depolarized and contracted. The postinfusion depression of the ST segment and inversion of the T wave may represent abnormal prolongation of the action potential; restoration of the electrocardiogram to normal by small doses of epinephrine or norepinephrine, substances which are known to shorten the action potential, support this view. Acetylcholine, which has little or no effect on the membrane or action potential of ventricular muscle produced transient effects similar to those observed with the catechol amines. The observations of Hoffman(8) and West (9) suggest that the changes induced by acetylcholine may have been the result of the stimulation of intracardiac ganglia and release of norepinephrine from the nerve endings. The mechanism by which procaine and phenoxybenzamine reversed the negative T wave is obscure. The lack of effect of these drugs on the normal electrocardiogram indicates that the reversal was not the result of direct peripheral action, but may represent the blockade of adrenergically induced vasoconstriction, as indicated in the recent reports of Sventivanyi(10).

The postinfusion depression of the ST segment and inversion of the T wave, persisting for 7-10 days, closely resembles the electrocardiographic pattern noted in patients with so-called myocardial ischemia(11,12). Numerous investigators have reported minor, transient ST segment and T wave alterations produced by fear or psychological stresses and have noted the similarities of these changes to those produced by parenteral administration of small doses of epinephrine (13). We are not aware of any previous report of postinfusion changes similar to those noted here. These observations may serve as a clue to the manner in which emotional factors may play a role in pathogenesis of coronary artery disease.

*Summary.* Infusion of epinephrine or norepinephrine into the coronary artery of dogs produced high, upright, peaked T waves with depressed ST segments. After cessation of the infusion the electrocardiogram approached normal, then gradually developed depressed and coved ST segments and inverted T waves which persisted for 7-10 days. The postinfusion electrocardiogram could be restored to normal by intracoronary infusion of epinephrine, norepinephrine, acetylcholine, procaine, or phenoxybenzamine. The persistent alteration of the electrocardiogram in our animals was remarkably similar to that attributed to subendocardial ischemia in man. No evidence of structural changes was noted in the heart of one dog sacrificed after several months of infusions.

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Received May 1, 1961. P.S.E.B.M., 1961, v107.

# The Specificity of Allergic Reactions. IV. The Cornea. (26662)

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Intracorneal injection of foreign protein into animals with delayed hypersensitivity to that protein produces damage to the cornea. This allergic reaction consists of edema, swelling, and necrosis of the corneal fibers; heavy polymorphonuclear leucocyte infiltration; and occasional necrosis of overlying epithelium. This reaction, occurring in the avascular cornea, might represent an antigen-specific cytotoxic response, independent of both blood vessels and circulating antibody. Moreover, Arthus reactions presumably could not occur in the cornea, even when reactions of this type are pronounced in the skin, since an Arthus reaction is associated with endothelial injury.

Marked changes in scarified corneas after instillation of nonhemolytic streptococci were described by Derick and Swift(1) in rabbits sensitized previously to this bacterial antigen (1). Julianelle(2,3) provoked similar corneal reactions in rabbits after sensitization with intact pneumococci, but he could not detect such responses in rabbits sensitized with pneumococcal nucleoprotein and having Arthus reactions to this antigen. He further showed that serum from rabbits hypersensitive to either egg albumin or pneumococcus nucleoprotein conferred Arthus reactions, but not corneal reactions, to normal recipients. Corneal reactions were then shown to be allied to tuberculin hypersensitivity(4,5), in that (a) the response required 24-48 hours to develop; (b) tuberculoprotein had a markedly toxic action on connective tissue of corneas of tuberculous guinea pigs, and led to inflammation and partial degeneration; and (c) histologically, the cornea showed changes characteristic of allergic responses.

A more recent work by Raffel *et al.*(6) describes the appearance of corneal reactions in guinea pigs after sensitization with a pure protein (*i.e.*, egg albumin) as long as tubercle bacillus "wax" was added to the sensitizing medium. Leskowitz and Waksman(7) have shown definite intracutaneous delayed reactions in rabbits after sensitization to bovine serum albumin in complete Freund's adjuvant as well as simultaneous corneal reactions to the same antigen. Schlossman and Stetson(8) have described vascularization of the cornea after intracorneal injections of antigen in guinea pigs sensitized with ovalbumin-anti-ovalbumin specific precipitates in complete Freund's adjuvant.

In the course of immunization of guinea pigs with purified proteins, delayed hypersensitivity preceded in time Arthus hypersensitivity and circulating antibody(9). As the animal progressed from a state of delayed allergy to one of antibody formation, a change in specificity to the antigen appeared, with delayed hypersensitivity oriented toward a broad portion of the antigenic molecule and Arthus reactions directed toward small, finite groupings(10,11,12). This observation suggests a method for determining whether corneal reactions are purely of the delayed type, whether they are influenced by circulating antibody, and to what portion of the antigenic molecule their specificity is directed. Accordingly, investigations were initiated in guinea pigs to elucidate further the exact relationship between corneal response, delayed hypersensitivity and circulating antibody.

*Materials and methods. Animals.* Guinea pigs were of the Hartley strain and weighed between 350-450 g.

*Antigens.* Five times recrystallized hen egg albumin (HEA) was obtained from K & K Laboratories, Inc., Jamaica, N. Y. Purified bovine gamma globulin (BGG) from Armour Laboratories, Chicago, Ill., was used without further treatment. Conjugated proteins were made as previously described(12).

*Sensitization.* The antigen was dissolved in physiologic saline containing 1% guinea pig serum and emulsified in a syringe with an equal volume of Freund's adjuvant (Difco), with mycobacteria (2 mg per animal). Except where specified, the animals were sensitized by injection of 0.5 ml of the oil-water emulsion into the footpads.

*Skin tests.* Guinea pigs were tested intradermally on the sides with 0.1 ml of antigen containing 50 μg/ml protein. Reactions were observed and diameters of areas of induration measured at 4 hours and at 18 to 24 hours after injection.

*Corneal tests.* Two to 3 drops of 0.5% Pontocaine hydrochloride (Winthrop Laboratories, New York City) were placed on the corneal surface of the guinea pig eye several minutes before testing. 0.01 ml of the antigenic solution containing 500 μg/ml protein was injected intracorneally with a 30-gauge needle attached to a ¼-ml tuberculin syringe. The injections resulted in a temporary clouding of the cornea which cleared in about 2 hours. Reactions were observed 4, 24 and 48 hours after injection. Several animals were sacrificed by intraperitoneal injection of Nembutal, the eyes were removed, fixed in Zenker's solution, and sections of the cornea stained with Harris's hematoxylin and eosin.

*Antibody production.* Guinea pigs were bled just prior to skin testing, and the sera assayed for antibody by the passive cutaneous anaphylaxis (PCA) reaction(13). In the PCA tests, 0.1 ml test serum was injected intradermally in the flank. Three hours later, 350 μg protein in 0.5 ml physiologic saline and 0.5 ml 1% Evans blue in physiologic saline were introduced intravenously. Fifteen to 30 minutes later, areas of pigmentation in the skin were measured and recorded.

*Results. Corneal reactions to purified proteins.* Guinea pigs were sensitized by injection in the footpads with 50 μg bovine gamma globulin (BGG) in Freund's adjuvant containing 2 mg mycobacteria. Animals were bled, skin-tested, and tested intracorneally daily from the 5th to the 21st day after sensitization, and irregularly thereafter. No guinea pig was tested on more than one occasion. At this dose level, delayed hypersensitivity to BGG was present on the 5th day after sensitization, and circulating antibody on the 7th day (Table I). Corneal reactions, on the other hand, did not appear consistently until the 12th day, well after delayed hypersensitivity first could be detected and at a time when delayed reactions in the skin of the flanks were masked by Arthus reactivity. This delay in time of manifestation of the corneal reactions may be explained on the basis (a) that additional time was required for delayed hypersensitivity to reach a degree of intensity wherein corneal responses could be observed, or (b) that circulating antibody may in some way be associated with the expression of corneal hypersensitivity.

TABLE I. Corneal, Delayed and Arthus Responses after Sensitization with 50 μg BGG in Complete Freund's Adjuvant.

| Time after sensitization (days) | No. of animals | Corneal response | Delayed intracut. response | Arthus response |
|---|---|---|---|---|
| 5 | 2 | 0/2† | 2/2 | 0/2 |
| 6 | 3 | 0/3 | 3/3 | |
| 7 | 6 | 1/6 | 2*/6 | 3/6 |
| 8 | 4 | 0/4 | 1*/4 | 2/4 |
| 9 | 4 | 2/4 | | 4/4 |
| 10 | 2 | 0/2 | | 2/2 |
| 11 | 5 | 0/5 | | 5/5 |
| 12 | 8 | 4/8 | | 8/8 |
| 13 | 6 | 3/6 | | 6/6 |
| 14 | 8 | 2/8 | | 8/8 |
| 15 | 6 | 2/6 | | 6/6 |
| 16 | 4 | 3/4 | | 4/4 |
| 17 | 1 | 1/1 | | 1/1 |
| 18 | 4 | 2/4 | | 4/4 |
| 19 | 3 | 3/3 | | 3/3 |
| 20 | 2 | 2/2 | | 2/2 |
| 21 | 4 | 3/4 | | 4/4 |
| 25 | 3 | 3/3 | | 3/3 |
| 28 | 5 | 5/5 | | 5/5 |

* These guinea pigs did not have circulating antibody.

† Numerator indicates No. of guinea pigs with positive reaction; denominator, No. of guinea pigs tested.

Guinea pigs were then sensitized with 5 μg hen egg albumin (HEA) in Freund's adjuvant plus 2 mg mycobacteria (Table II). Herein, both delayed intracutaneous and corneal reactions appeared on days 4-5, whereas circulating antibody could not be detected until days 8-9. Thus, corneal reactions were not associated with the appearance of circulating antibody, as determined by PCA tests which can detect 0.001-0.002 μg antibody nitrogen.

TABLE II. Corneal, Delayed and Arthus Responses after Sensitization with 5 μg HEA in Complete Freund's Adjuvant.

| Time after sensitization (days) | No. of animals | Corneal response | Delayed response | Arthus response |
|---|---|---|---|---|
| 4 | 4 | 4/4 | 4/4 | |
| 5 | 5 | 4/5 | 5/5 | |
| 6 | 5 | 4/5 | 5/5 | |
| 7 | 5 | 5/5 | 5/5 | |
| 8 | 3 | 2/3 | | 3/3 |
| 9 | 3 | 3/3 | | 3/3 |
| 10 | 2 | 2/2 | | 2/2 |
| 11 | 4 | 3/4 | | 4/4 |
| 13 | 2 | 2/2 | | 2/2 |
| 18 | 3 | 3/3 | | 3/3 |
| 19 | 4 | 1/4 | | 4/4 |
| 20 | 2 | 2/2 | | 2/2 |

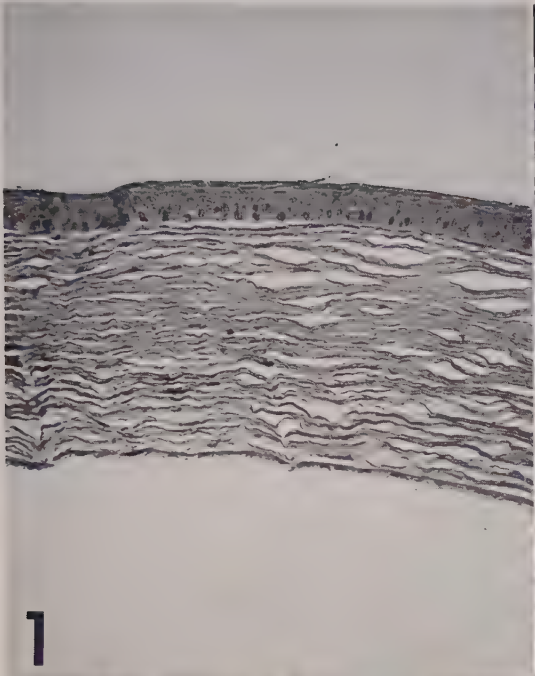
The corneas from the hypersensitive animals in experiments with both BGG and HEA were markedly thickened and opaque. Histological sections showed that the bands of sensitized corneal fibers had thickened to about twice the cross-section of the bands of normal fibers, and that the corneas had extensive cellular exudation and striking polymorphonuclear infiltration (Fig. 1-4).

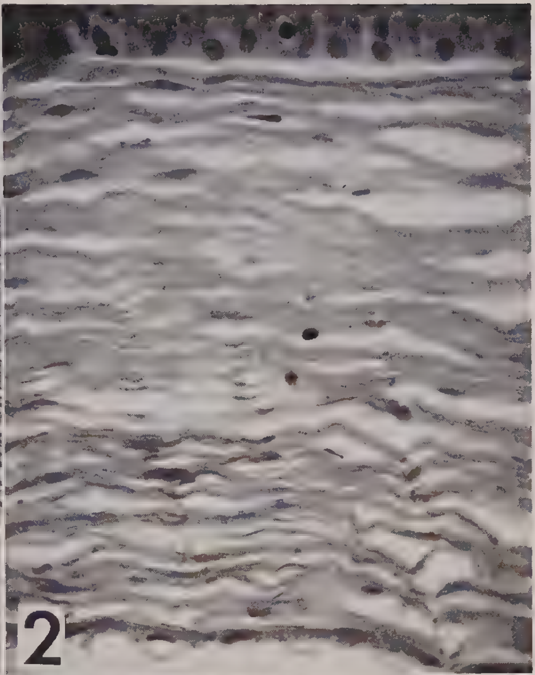
The animal that tended to show a stronger intradermal delayed response also tended to have the more pronounced corneal reaction. Frequently, however, guinea pigs with weak to moderate delayed intradermal responses on the sides did not develop visible corneal opacities. Thus, if both corneal and intracutaneous delayed reactions are essentially caused by the same mechanism, the corneal tissue shows allergic changes less readily.

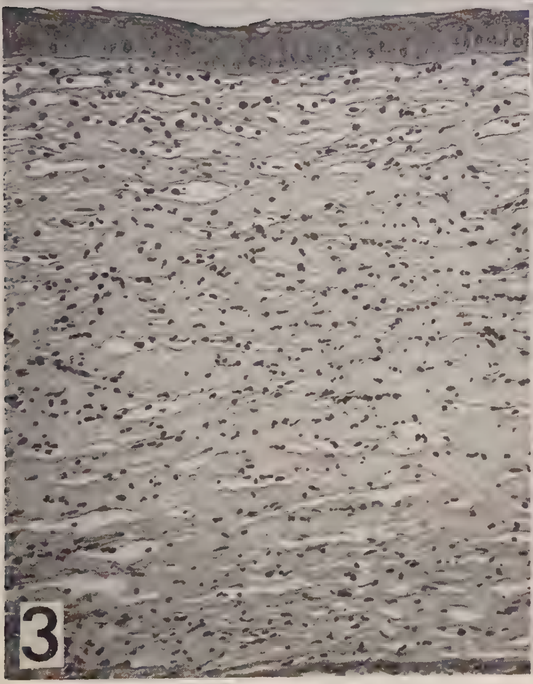
Twenty-four guinea pigs were sensitized in the footpads with 50 μg BGG in Freund's adjuvant without mycobacteria, and tested periodically up to 21 days postsensitization for cutaneous delayed hypersensitivity and for corneal hypersensitivity. Delayed reactions occurred after intradermal injections, but allergic reactions could not be detected after intracorneal testing. Twenty-one additional guinea pigs were sensitized with 1.0 μg HEA in saline in the footpads and 10 days later administered a second dose of 5.0 μg HEA in Freund's adjuvant (without mycobacteria)(14). Periodic testing up to 23 days postsensitization again produced no evidence of corneal hypersensitivity. The tubercle bacillus apparently increases the hypersensitivity sufficiently so that the cornea can be affected or adds a substance to the tissues which enables them to react. The former concept seems more logical.

*Specificity.* Guinea pigs sensitized with para-amino benzoic acid diazotized to hen egg albumin (PABA · HEA) develop initially a delayed state (as indicated by intracutaneous testing) which is oriented in its specificity primarily toward the HEA part of the antigen molecule, whereas the later-appearing Arthus phase is directed primarily toward the PABA moiety(12). Since the corneas, from 4 to 45 days after sensitization, required from 24 to 48 hours for the allergic opacity to become macroscopically visible, delayed hypersensitivity seemed to be the basis for this immune reaction. No change in the appearance of the reaction occurred in the cornea during this 4- to 45-day period, although the skin was undergoing a change from delayed to Arthus reactions. To determine whether this consistency in the corneal reaction was apparent or real, corneal reactions were examined in guinea pigs sensitized to PABA · HEA.

Guinea pigs were sensitized in the footpads by injection of 15 μg PABA · HEA in Freund's adjuvant plus 2 mg tubercle bacillus. Pairs of guinea pigs were then tested in the cornea with either PABA · HEA and HEA, PABA · HEA and PAAA · HEA (p-amino arsonic acid diazotized to HEA) or PABA · HEA and PABA · BGG (p-amino benzoic acid diazotized to bovine gamma globulin) and at the same time intradermally with PABA · HEA (Table III). The specificity pattern of delayed hypersensitivity in the cornea consistently followed that typical of the skin. For as long as 45 days after sensitization, a reaction followed intracorneal injection of PABA · HEA, HEA or PAAA · HEA. At no time, however, did a corneal reaction appear after introduction of the homologous hapten-heterologous protein conjugate, PABA · BGG, even though circulating antibody was present and Arthus reactions could be elicited to PABA · BGG. Thus, for 45 days after sensitization, a pure delayed reaction can develop in the cornea,







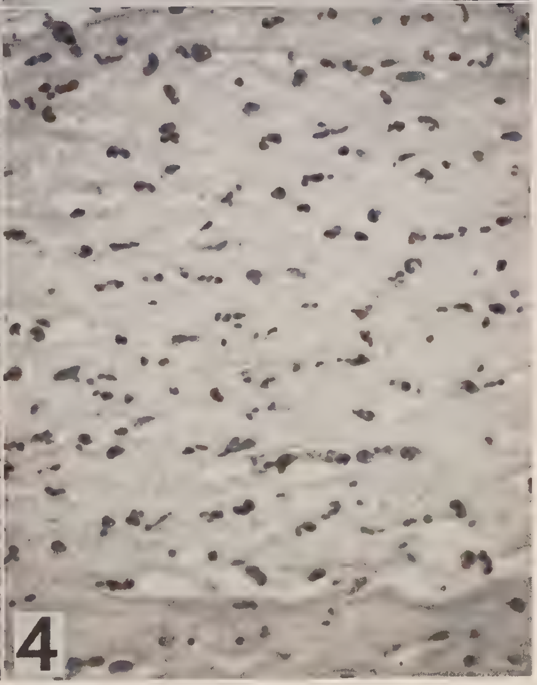


FIG. 1. Section of cornea from a nonsensitized guinea pig 24 hr after intracorneal inj. of 5 $\mu$g hen egg albumin. × 187.

FIG. 2. Higher magnification of same section of cornea as in Fig. 1. × 461.

FIG. 3. Section of cornea from a guinea pig sensitized 19 days previously with 5 $\mu$g HEA in complete Freund's adjuvant 24 hr after intracorneal inj. of 5 $\mu$g HEA. × 187.

FIG. 4. Higher magnification of same section of cornea as in Fig. 3. × 461.

TABLE III. Corneal, Delayed and Arthus Reactions after Sensitization with 15 $\mu g$ PABA • HEA in Complete Freund's Adjuvant.

| Time after sensitization (days) | No. of animals | Corneal response to | | | | Intradermal response to PABA • HEA | |
|---|---|---|---|---|---|---|---|
| | | PABA • HEA | PABA • BGG | HEA | PAAA • HEA | Delayed | Arthus |
| 6 | 2 | 2/2 | | 2/2 | | 2/2 | |
| 7 | 1 | 1/1 | | 1/1 | | 1/1 | |
| 8 | 7 | 6/7 | 0/3 | 3/4 | | 7/7 | |
| 9 | 2 | 2/2 | | 1/2 | | 2/2 | |
| 10 | 4 | 1/4 | 0/2 | 1/2 | | 4/4 | |
| 12 | 7 | 6/7 | 0/2 | 4/5 | | 4/7 | 3/7 |
| 13 | 4 | 3/4 | 0/2 | 1/2 | | 2/4 | 2/4 |
| 14 | 4 | 1/4 | 0/2 | 1/2 | | 3/4 | 1/4 |
| 15 | 4 | 3/4 | 0/2 | 1/2 | | 2/4 | 2/4 |
| 16 | 3 | 3/3 | 0/1 | 0/2 | | 1/3 | 2/3 |
| 19 | 7 | 4/7 | 0/3 | 0/4 | | | 7/7 |
| 26 | 4 | 3/4 | 0/2 | 2/2 | | | 3/4 |
| 29 | 2 | 2/2 | 0/1 | 0/1 | | | 2/2 |
| 32 | 4 | 2/4 | 0/2 | 0/2 | | | 4/4 |
| 39 | 6 | 5/6 | 0/3 | | 3/3 | | 6/6 |
| 45 | 4 | 4/4 | 0/2 | | 2/2 | | 4/4 |

in spite of the possible presence of circulating antibody, as judged by the foregoing experiments with protein conjugates.

*Discussion.* Introduction of minute quantities of antigen in Freund's adjuvant into the footpads of guinea pigs results in delayed hypersensitivity, followed by appearance of circulating antibody. The delayed hypersensitivity may also be observed after transfer of washed lymph node cells from an allergic animal to a normal one and after subsequent intradermal testing of the recipient (9). During this period of delayed allergy, circulating antibody to the allergen cannot be detected.

During much of the time in which delayed allergy can be demonstrated in the skin and also during the subsequent period when circulating antibody can be demonstrated by PCA tests and Arthus reactions, injection of the cornea with specific antigen induces a reaction. This response seems to be basically controlled by the mechanism associated with delayed hypersensitivity. Even though circulating antibody and Arthus reactions may be present, and even though some vascularization may develop in the fibrous layer of the cornea, the specificity of the response is oriented toward the protein moiety of a conjugated antigen. The corneal tissue does not respond to circulating antibody, or possibly the concentration of gamma globulin in the cornea does not reach or exceed threshhold values. That the former hypothesis seems to be the true one is further indicated by the fact that guinea pigs with delayed hypersensitivity to pure proteins (HEA) or tuberculin showed a delayed polymorphonuclear response in the cornea after intravitreal challenge, although other ocular tissues such as the choroid, sclera, and uvea, show predominantly a mononuclear infiltration(15). Injection of antibody intravitreally followed by intracorneal testing causes an iridocyclitis more readily than a corneal opacity(16). Passive transfer of sensitized cells intravenously into normal recipients produces a delayed hypersensitivity which can be detected by corneal injections(17).

The corneal response, in contrast to the intradermal delayed reaction, seems to be primarily polymorphonuclear in cell type. Whether this is actually so, or whether the polymorphonuclear invasion follows one of mononuclear elements(18), requires further investigation. A similar polymorphonuclear infiltration was demonstrated on subarachnoid injection of "purified protein derivative" of the tubercle bacillus into tuberculin positive humans(19). Also, explants of cornea from a sensitized guinea pig do not show a cytotoxic response when they are exposed to specific antigen in tissue culture (20). This observation seems to point toward invading leucocytes as the cells responding chemotactically to injection of antigen in the cornea. The possibility arises, therefore, that the cells of the cornea are merely the seat of the allergic response, and play either a minor role or none at all in actually eliciting it.

*Summary*. Injection of protein antigens in complete Freund's adjuvant into the footpads of guinea pigs produces a hypersensitivity which induces allergic reactions in the cornea both during the delayed and Arthus phases of the allergic response. This corneal reaction is similar to the intracutaneous delayed type in that its specificity is oriented toward the protein portion of a conjugate and not toward the hapten.

The authors express their sincere appreciation to Jane Nishio, Aspascia Cobure and LeRoy F. Peel for technical assistance.

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Received May 4, 1961. P.S.E.B.M., 1961, v107.

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## Fatty Acids of Human Platelet Phosphatides.* (26663)

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The development of gas-liquid chromatography (GLC) provides a highly sensitive method for separation of fatty acid mixtures and identification of individual components (1,2). Studies of the fatty acid composition of a wide variety of lipids have been reported (3,4). Since platelet phosphatides exhibit specialized activity in the generation of blood thromboplastin(5) it is of interest to determine their constituent fatty acids by means of GLC.

*Materials and methods*. Blood was collected(5) for platelet isolation from 25 subjects without apparent hematologic disorder. The phospholipids were extracted(5) and pooled. One hundred fifty-nine mg of crude phospholipid were chromatographed on a silicic acid column and the 150 individual fractions identified by paper chromatography(5). Each fraction was esterified by transmethylation in superdry methanol followed by sublimation according to Stoffel *et al.*(6). The yield of methyl esters from the sublimation cold finger was at least 95%

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* This investigation was supported by a grant from N. Y. Heart Assn.

and colorless in petroleum ether solution. The GLC pattern of each silicic acid column fraction was determined with a Barber-Colman Model 10 chromatograph, equipped with a radium detector cell operated at 1000 volts, and a sensitivity of 1 × $10^{-7}$ amps. The methyl esters derived from the ascending and descending limbs of the 2 main silicic acid column peaks were pooled since they showed similar patterns. Argon provided the mobile phase at an inlet pressure of 35 psi with a flow rate at the column outlet of 200 ml per minute. To remove contaminants in the carrier gas, the tubing from the argon tank to the column was filled with 18 g of Molecular Sieve type 5-A(7). A U-shaped pyrex glass column, 6 feet in overall length with an internal diameter of 4 to 5 mm was employed. The stationary phases were adipate and succinate polyesters of ethylene glycol, 13.3% and 15% by weight respectively on 80-100 mesh Chromosorb W[†]. The "flash heater" was operated at a temperature of 250°C and detector cell at 230°C. The adipate column was held at 173°C, the succinate column at 180°C. Each column was conditioned at 197°C using a pressure of 40 psi for 4 days to minimize "bleeding." Percentage composition of the column eluate was determined by triangulation(1). Linearity of the detector response was evaluated by examining eluates of known purified fatty acid methyl esters.[†] In general, a difference of from 2 to 6% was noted between the amount applied and that eluted from the column. The chain length of fatty acids and their degree of unsaturation was determined by comparison with known standards and the retention times relative to methyl stearate, as reported by Farquhar *et al.*(1).

Since phosphatidylethanolamine (PE) and phosphatidylserine (PS) could not be separated adequately on the silicic acid column, the methyl esters of these phosphatides were examined together. PE and PS have previously been shown to represent the fraction of the platelet phospholipid active in clotting (8). Lecithin and sphingomyelin were also incompletely resolved from each other and studied as a mixture. They do not possess clot promoting activity(8).

*Results.* The pattern of the silicic acid column separation follows our previous reports(5,8). PE and PS predominate in the first 20 fractions, whereas lecithin and sphingomyelin form the bulk of tubes 21-150. The results of GLC on the methyl esters of the pooled tubes 7-10 and 11-16 are shown in Table I. These represent fractions on the ascending and descending limb of the PE-PS peak; tube numbers 27-35 and 36-150 in the Table are similar areas in the lecithin-sphingomyelin elution. Only the findings with adipate polyester at 173°C are given, but results with succinate polyester are similar. The adipate column had an efficiency of 2400 theoretical plates at methyl stearate (retention time 33 minutes). To simplify nomenclature, the shorthand designation(9) has been employed. Although approximately 37 components can be resolved in each chromatogram, many are minor and cannot be identified. These account for less than 2% of the total, and for purposes of clarity, are not shown in the Table. Values are reported as per cent of total methyl esters.

The principal fatty acids in the PE-PS fractions are arachidonic (45, 43%), stearic (22, 27%) and oleic (19, 13%). The lecithin-sphingomyelin elution shows essentially 2 patterns: In the early phase the primary fatty acids are oleic (45%), palmitic (23%), and stearic (18%) with a limited amount of linoleic (4%). These proportions change in the descending limb of the peak so that palmitic acid (64%) predominates; oleic (16%), stearic (6%) and linoleic (1%) decrease markedly. Distribution of the major fatty acids in the platelet phospholipids is shown in Fig. 1.

The dimethyl acetal derivatives of tetradecanal, hexadecanal, and octadecanal are noted in the PE and PS fractions. These represent the acetals formed on methylation of the aldehydogenic unit of plasmalogens (10). These phosphatides were previously noted to occur mainly in the PE and PS peaks and only trace amounts were detected

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[†] Obtained from Applied Science Laboratories, State College, Pa.

TABLE I. Gas Chromatography of Platelet Phosphatide Fatty Acids Using EGA Polyester at 173°C as Stationary Phase.

| Familiar name | Shorthand designation | Retention time relative to methyl stearate | % of total fatty acids: PE-PS fractions 7–10 | 11–16 | Lecithin-sphingomyelin fractions 27–35 | 36–150 |
|---|---|---|---|---|---|---|
| Lauric | 12:0 | .137 | .2 | .3 | .3 | .3 |
| | Unknown | .199 | .4 | .5 | .5 | 1.3 |
| | 14:0 DMA* | .222 | 1.1 | 1.8 | .2 | .6 |
| Myristic | 14:0 | .274 | .8 | 1.3 | .2 | .2 |
| | 16:0 DMA | .430 | 2.3 | 3.1 | .05 | .1 |
| Palmitic | 16:0 | .496 | 1.1 | 1.1 | 23.8 | 64.1 |
| Palmitoleic | 16:1 | .550 | 1.1 | 2.2 | .4 | .51 |
| | Unknown | .610 | .2 | .3 | .1 | .24 |
| | 17:0 | .695 | .1 | .1 | .2 | .26 |
| | Unknown | .800 | .2 | .4 | .03 | — |
| | 18:0 DMA | .900 | .1 | .1 | .02 | — |
| Stearic | 18:0 | 1.00 | 22.4 | 27.7 | 18.6 | 6.5 |
| Oleic | 18:1 | 1.12 | 19.7 | 13.8 | 45.0 | 16.4 |
| Linoleic | 18:2 | 1.36 | 2.1 | 1.2 | 4.5 | 1.8 |
| Arachidic | 20:0 | 1.97 | .2 | .2 | .8 | 1.3 |
| | 20:1 | 2.16 | .2 | .1 | .6 | .7 |
| | 20:3 | 3.00 | .2 | .3 | .4 | — |
| Arachidonic | 20:4 | 3.32 | 45.6 | 43.5 | 3.1 | 1.8 |
| | 20:unsat. | 3.87 | .2 | — | .8 | 3.2 |
| | 20:5 | 4.22 | .3 | .1 | .2 | — |
| | 22:unsat. | 6.50 | .8 | 1.0 | .2 | — |
| | 22:5 | 8.35 | .3 | .4 | .3 | .7 |
| | 22:6 | 9.30 | .4 | .5 | — | — |

* Dimethyl acetal of fatty aldehyde.

in the lecithin-sphingomyelin fractions(5, 11).

*Discussion.* The PE-PS fractions of platelet phosphatides can substitute *in vitro* for the entire platelet in formation of blood thromboplastin(5,8). Arachidonic acid represents approximately one-half of the fatty acid moiety in these eluates. Stearic and oleic acids are also major constituents of platelet PE and PS. The association of plasmalogens with the fractions promoting thromboplastic activity is again noted(5). In the lecithin-sphingomyelin peak, arachidonic acid virtually disappears. This striking reduction is of interest since clotting activity is absent in these fractions(5,8). Although it has been generally assumed that the ethanolamine or serine residue is responsible for the clotting activity of platelet "cephalin"(5), these results suggest that fatty acid moiety may be of considerable or even primary importance. Studies on the relationship of unsaturated fatty acid moieties to thromboplastic activity have been reported by Rouser and Schloredt(12) and Wallach *et al.*(13). When synthetic phosphatidylethanolamine containing 2 saturated fatty acids (L-alpha-(dimyristoyl) cephalin)[‡] was

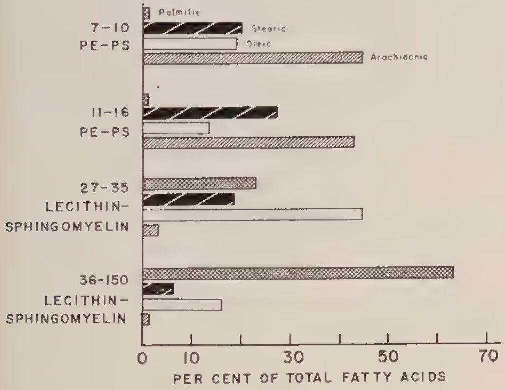

FIG. 1. Distribution of major fatty acids in blood platelet phosphatides as separated by silicic acid and gas-liquid chromatography. Fractions 7-10 and 11-16 are active in formation of blood thromboplastin, whereas 27-35 and 36-150 are not.

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[‡] Kindly provided by Dr. Erich Baer.

tested in the thromboplastin generation test, it was completely inert in all concentrations studied. It must also be pointed out that the exact relationship of plasmalogens to blood coagulation has not been clarified.

The results of GLC analyses of fatty acid composition of red cell phosphatides recently made by Farquhar *et al.* show an overall similarity to the patterns of platelets.§ This is of importance because it has been found that red cell phospholipids can promote formation of blood thromboplastin(14,15).

*Summary.* The fatty acids of blood platelet phosphatides have ben studied by silicic acid, and gas-liquid chromatography. PE-PS fractions contain primarily arachidonic, stearic and oleic acids. Lecithin-sphingomyelin eluates contain principally palmitic, stearic and oleic acids. These components may be quantitatively related to the specialized function of platelets in blood coagulation.

The authors wish to thank the following individuals for advice and encouragement in this work: Doctors John W. Farquhar, S. R. Lipsky, Helen Anderson, Theodore H. Spaet, and J. James Smith.

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Received March 24, 1961. P.S.E.B.M., 1961, v107.

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# Serologic Studies of Rabbit Antibodies to Rabbit Submaxillary Glands.* (26664)

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Experimental evidence has accumulated during the last few years in reference to the pathogenesis of chronic non-specific thyroiditis(1). It has been shown that experimental animals such as rabbits, dogs and guinea pigs when immunized with extracts of thyroid glands of the respective species frequently develop thyroid specific antibodies demonstrable either by the usual serological tests as circulating antibodies or by technics employed to demonstrate delayed hypersensitivity, or both(2). They also frequently show histological evidence of thyroiditis.

Recently attention has been drawn to the pathogenesis of Sjögren's syndrome, a clinical triad characterized by keratoconjunctivitis sicca, xerostomia and also rheumatoid

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* This work was supported in part by a research grant from Richard W. and Mae Stone Goode.

arthritis(3,4,5). Some of the same clinical characteristics have been reported in some patients with lupus erythematosus and scleroderma. Bloch and associates(5) have reported results of different serological tests with sera of patients suffering from Sjögren's syndrome, and have referred to the pathology of the salivary gland. Extensive accumulation of lymphocytes and plasma cells was observed in biopsy material obtained from the parotid or submaxillary glands of 10 patients. Experiments have been carried out in our laboratory to determine whether it would be possible to find an organ-specific antigen in the submaxillary and related glands of rabbits; whether that antigen would prove to be antigenic for rabbits and finally, whether lesions would occur in the glands of experimental rabbits which would resemble those seen in Sjögren's disease.

*Materials and methods.* The submaxillary gland of normal rabbits was removed and immediately frozen in liquid nitrogen. Suspensions for immunization purposes were prepared by grinding 1 part of submaxillary gland and 4 parts of buffered saline in a Waring blendor. After blending for about 60 seconds the emulsified material was passed through a plastic screen and stored in the freezer until ready for use. Buffered saline extracts were prepared by centrifuging 10% suspensions of rabbit organs at about 20,000 g for 10 minutes. These suspensions were prepared by homogenizing 1 part of rabbit organ with 9 parts of buffered saline in a Waring blendor. For preparation of smaller amounts of suspension and extract, a glass hand homogenizer was used. Extracts were kept in a deep freeze until ready for use.

New Zealand white rabbits were injected intradermally in the 4 footpads and in several sites in the neck region with a total of 1 cc of a mixture containing equal parts of 20% suspension of rabbit submaxillary gland and complete Freund adjuvant. Mycobacteria used in the adjuvants were prepared according to the method of Colover(6): virulent human tubercle bacilli (strain H37 Rv) are heat killed at 100°C for 30 minutes and extracted in a Soxhlet, first with acetone and then with isopropyl ether. The dried residue is incorporated into incomplete Freund adjuvant, 5 mg per 10 ml. There were 2 groups of rabbits. The first group of 4 rabbits received 2 sets of injections at an 8 week interval. The serological results reported here were obtained with terminal bleedings taken 2 weeks after second injection. A second group of 3 rabbits received a single set of intradermal injections and were bled 3 weeks afterward.

*Complement fixation* was carried out by mixing 0.1 ml of antiserum dilution, 0.1 ml of antigen dilution, and 0.1 ml of guinea pig complement (diluted 1:16 or 1:18). The mixtures were kept for 18 hours in the refrigerator at 4°C, then for 1 hour at 37°C. 0.2 ml of sheep cells sensitized with appropriate dilution of amboceptor was added. Several readings were made, first after the controls had cleared (usually after 15-20 min) and final readings 40-55 min later. Only complete inhibition of hemolysis after clearing of controls was considered as positive.

*The tanned cell hemagglutination test* was carried out using essentially the technic employed in the thyroglobulin studies reported previously(7). The procedure in the present study was as follows: 1) washing human group O Rh positive red cells with pH 7.2 phosphate buffered saline 4 times, 2) tanning cells by mixing a 4% suspension with an equal volume of 1:30,000 tannic acid for 30 minutes, 3) washing 4 times with buffered saline, 4) coating with rabbit submaxillary globulins kept in a boiling water bath for 4 minutes; dilutions of the globulin ranged from 1:50 to 1:1,000, 5) washing 3 times with a 1/100 buffered saline dilution of normal rabbit serum. Antiserum dilutions were also prepared with 1/100 buffered saline diluted normal rabbit serum. Tests were carried out by mixing 0.1 ml of 1.5% coated cell suspensions with 0.1 ml of antiserum dilutions. Readings were based upon observations of the agglutination pattern after standing at room temperature for several hours, refrigerating overnight, and standing for 3 hours. Some of the positive submaxillary antisera exhibited a certain degree of agglutination in dilutions of 1:10 to 1:20 when the tubes were shaken at the end of ob-

TABLE I. Specificity of Rabbit Submaxillary Gland Antisera Tested against Extracts of Organs of Rabbit #1565.

| Antisera, 1:15 dilution | Last extract dilution giving positive reaction | | | | | |
|---|---|---|---|---|---|---|
| | Submaxillary | Parotid | Adrenal | Harderian | Liver | Kidney |
| #1565 | >2700 | >2700 | 30 | <30 | 30 | 100 |
| #1570 | 900 | <30 | 30 | 30 | 30 | 30 |
| #1001 control* | 30 | <30 | 30 | 30 | 100 | 30 |

* Treated with 2 series of injections of complete Freund adjuvant only.

servation time. (The tanned, but uncoated cells were negative). Agglutination readings summarized in Table III represent *patterns* of sedimentation rather than clumping agglutination observed on shaking.

The insoluble material mentioned in Table II was prepared by first making a 10% suspension in buffered saline, centrifuging at about 30,000 g for 10 minutes removing the supernatant extract and resuspending the sediment in 0.25 M sucrose. The suspension was then subjected to differential centrifugation according to the method of Hogeboom and Schneider(8) for isolation of nuclear, mitochondrial and microsomal fractions. The water-soluble extracts were divided into globulins and albumins by precipitation with 50% saturated ammonium sulfate. The washed globulin precipitates were reconstituted to the volume of the original 10% organ extracts by dissolving in phosphate buffered saline, used throughout these studies, made up of 0.115 N sodium chloride and an 0.035 N mixture of sodium and potassium phosphate adjusted to pH 7.2.

*Results.* The antisera obtained by immunization of rabbits with suspensions of pooled freshly frozen submaxillary glands incorporated into Freund adjuvant were examined for presence of circulating antibodies by several serological technics. There was no basic difference in the results between the 2 series, one of which had received one injection, the other series, two injections. Three out of 7 animals revealed the presence of antibodies by means of complement fixation and 6 in the hemagglutination test. The 3 antisera containing antibodies against saline extracts of pooled rabbit submaxillary gland failed to react significantly with saline extracts of other organs. The question arose whether the antibodies produced by isoimmunization would also act on extracts of glands of the antibody-producing animal. A typical experiment is shown in Table I.

Rabbit antiserum #1565 when tested against organ extracts obtained from the same animal fixed complement with extracts of the submaxillary gland and the parotid gland but not with extracts of 4 other organs (Table I). Antiserum #1570 reacted exclusively with the extract of the submaxillary gland but not with the parotid extract nor with the other organ extracs of animal #1565. Aniserum #1001 used as a control shows that the extracts of the submaxillary glands do not fix complement *per se* when tested with rabbit sera. Therefore, the antibodies produced by isoimmunization of rabbits with extracts of the submaxillary gland are of considerable specificity. An additional antiserum #1564 exhibited identical reactivity to that of #1570; namely, it reacted with submaxillary gland but not with other organ extract. In other tests, no reactivity was demonstrable with lung extracts. In tests with extracts of rabbit, cat, and human submaxillary extracts these sera (1565 and 1570) exhibited significant reactivity only with the homologous (rabbit) extract.

Information on the nature of the antigen involved was sought by testing various fractions of the submaxillary glands. Table II summarizes the complement fixing activity of 2 of the antisera, #1565 and #1570, with

TABLE II. Complement Fixation Tests with Submaxillary Gland Components and Rabbit Antibodies to Rabbit Submaxillary Gland.

| Antigens (components of rabbit submaxillary) | Percentage of reactivity of 10% extract | |
|---|---|---|
| | 1565 | 1570 |
| 10% extract | 100 | 100 |
| Globulins | 100 | 100 |
| Albumins | <1 | <1 |
| Microsomes | 25 | 25 |
| Mitochondria | <10 | <10 |
| Nuclei | <10 | <10 |

TABLE III. Tanned Cell Hemagglutination with Rabbit Submaxillary Gland Globulin and Rabbit Thyroglobulin Coated Cells.

| Serum dilutions | Rabbit sera | | | | | |
|---|---|---|---|---|---|---|
| | NRS | 1214 (F.A.) | 1565 (R.Sm.) | 1570 (R.Sm.) | 1364 (R.Th.) | 1365 (R.Th.) |
| | Rabbit submaxillary globulin coated cells | | | | | |
| 1:10 | — | — | ++++ | ++++ | — | — |
| 1:50 | — | — | ++++ | +++ | — | — |
| 1:250 | — | — | +++ | + | — | — |
| 1:1250 | — | — | + | — | — | — |
| 1:6250 | — | — | ± | — | — | — |
| 1:31 × $10^3$ | — | — | — | — | — | — |
| 1:10 u.c. | — | — | — | — | — | — |
| | Rabbit thyroglobulin coated cells | | | | | |
| 1:10 | — | — | — | — | ++++ | ++++ |
| 1:50 | — | — | — | — | ++++ | ++++ |
| 1:250 | — | — | — | — | +++ | ++++ |
| 1:1250 | — | — | — | — | +++ | + |
| 1:6250 | — | — | — | — | ++ | ? |
| 1:31 × $10^3$ | — | — | — | — | — | — |
| 1:10 u.c. | — | — | — | — | — | — |

NRS = Normal rabbit serum.
1214 = Rabbit antiserum to Freund adjuvant emulsified in buffered saline, 3 groups of injections.
1565, 1570 = Rabbit submaxillary gland antibodies, see Table I.
1364, 1365 = Rabbit thyroglobulin antibodies.

the microsomal, mitochondrial and nuclear fractions of the insoluble components of submaxillary suspensions as well as the albumin and globulin fractions of the soluble part.

Fractions of insoluble components were tested at and beyond the limits of the anticomplementary activity. Results were calculated back to the volume of the original 10% suspension (in 0.25M sucrose) for comparison with activity of the 10% extract. Fractions of soluble components prepared by 50% saturated ammonium sulfate precipitation of globulins were adjusted to the volume of the original 10% buffered saline extract. Relative reactivity of submaxillary gland components in terms of reactivity of the crude extract is shown in Table II.

Virtually all of the complement fixing activity appears to reside in the globulin fraction of the extract. Some reactivity was demonstrable in the microsomal fraction, which may be explained by the fact that protein synthesis is considered to be carried out primarily by the microsomal components. Antigenic activity of the microsomes is not included with the activity of the extract because the microsomes are probably not included in the extract as they were very likely sedimented in its preparation. Thus, the sum total of the antigenic activities of the submaxillary gland components appears to be greater than 100%.

Table III summarizes results obtained when rabbit submaxillary antisera #1565 and #1570 were examined for tanned cell hemagglutination activity. For that purpose tanned Rh positive cells of Group O were coated with isolated globulin fraction of rabbit submaxillary gland. The specificity of the reatcion is demonstrated by simultaneous testing with tanned cells coated with isolated rabbit thyroglobulin.

The 2 submaxillary gland rabbit antisera #1565 and #1570 agglutinated tanned cells coated with globulin prepared from pooled rabbit submaxillary gland, but failed to agglutinate tanned cells coated with thyroglobulin (Table III). On the other hand, 2 rabbit antisera immunized with rabbit thyroglobulin agglutinated tanned cells coated with thyroglobulin but not with the globulin of the submaxillary gland. The specificity of the reaction was further examined by means of inhibition of the tanned cell hemagglutination test. For that purpose antiserum #1565 was mixed with varying dilutions of organ extracts, incubated for 1 hour at room temperature, then tested for its activity in the tanned cell agglutination test. The agglutinating capacity of this antiserum was inhibited by both the rabbit submaxillary extract and the rabbit parotid extract but not by the extract of the rabbit lung or rabbit thyroid. In similar inhibition experiments carried out with the 2 other antisera, #1564 and #1570, the submaxillary extracts exclusively inhibited the agglutination. These results confirm the observations on the spectrum of specificity of these 3 antisera recorded in Table I. Extracts of the submaxillary glands of the antibody producing rabbit inhibited hemagglutination as well as did submaxillary gland extracts of other rabbits.

*Discussion.* From previous investigations it was learned that there are at least 2 methods for considering the presence of experimentally-produced autoantibodies: (1) by immunizing thyroidectomized rabbits with extracts of the rabbit's own thyroid gland, and (2) by isoimmunization of a normal rabbit using thyroid extracts prepared from pooled thyroid glands of different rabbits. In the latter case antibodies obtained should react to the same extent with extracts of all individual rabbit thyroid glands examined, including that of the antibody-producing animal itself. Also, histological changes might be found in the thyroid gland of the antibody-producing animal which then could be considered as an effect of active immunization, (but by no means necessarily an effect of the circulating type of antibodies). In the experiments reported herein evidence for the appearance of antibodies against constituents of the salivary glands of rabbits is presented. So far, histological changes are not demonstrable to our satisfaction in the submaxillary glands of the antibody-producing animal. However, as in the field of autosensitization, histological changes sometimes appear only after a prolonged period of immunization and frequently are preceded by appearance of circulating antibodies. Therefore, this is being further investigated.

These observations might be of interest in human diseases of salivary glands. Urbain and Caroli(4) and others(5) suggest an "autoimmune" etiology of Sjögren's syndrome and related diseases of the salivary and lacrimal glands. Jones(3) reported demonstration of antibodies in sera of patients exhibiting Sjögren's syndrome which precipitate salivary gland extracts. Similar reactions, however, were demonstrated with kidney extracts but not with liver or muscle extracts. More recently, Bloch *et al.*(5) reported briefly on complement fixation tests carried out by Zvaifler and Bloch with Sjögren's syndrome sera and human organ suspensions in which complement was fixed not only in the presence of submaxillary gland suspensions but also with liver, kidney, or striated muscle suspensions. In view of the common association of nuclear autoantibodies with Sjögren's syndrome(5), it seems possible that the observed lack of specificity may be associated with antibodies fixing complement in presence of nuclear antigens. We are now studying the problem whether circulating antibodies specific for the glands involved in Sjögren's syndrome are demonstrable in human sera in addition to non-organ specific antibodies such as nuclear antibodies.

*Summary.* Sera of rabbits immunized with pooled rabbit submaxillary gland suspensions were studied by complement fixation and tanned cell hemagglutination technics. 1. Some of these antisera (3 out of 7) fixed complement in presence of rabbit submaxillary gland extracts. 2. Complement fixing activity of 2 of the 3 sera studied in detail was specific for submaxillary gland extracts within the limits of antigens tested. One of 3 serologically active sera (#1565) cross reacted with parotid gland extracts. 3. The globulin fraction of submaxillary gland extracts contained virtually all the material in the extract which was reactive with rabbit submaxillary gland antibodies. Some activity was also found in the microsome fraction. 4. Extracts of the submaxillary glands of the antibody-producing animals themselves reacted just as strongly as those of other rabbits, indicating the autoantibody nature of the antibodies elicited by isoimmunization.

5. Most of the rabbit antisera (6 out of 7) agglutinated tanned red cells coated with rabbit submaxillary globulin. 6. By hemagglutination inhibition tests, one antiserum (#1565) was found to be inhibited by submaxillary and parotid extracts. The other antisera tested were specific for submaxillary gland antigens. These results agree with those obtained by complement fixation.

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Received January 9, 1961. P.S.E.B.M., 1961, v107.

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## Production of Glucagon Antibodies and their Role in Metabolism and Immunoassay of Glucagon.* (26665)

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Lack of specificity is a significant disadvantage of certain technics for bioassay of glucagon(1-4). Recently developed methods for immunochemical assay of insulin(5,6) appeared adaptable to the specific measurement of glucagon. This report describes the development of antibodies to glucagon in rabbits treated during a 22-month period, the influence of the antibodies on the metabolism of glucagon and their potential use for immunochemical assay of this hormone. Recently, an independent study of similar nature was reported by Unger *et al.*(7).

*Methods.* Recrystallized porcine glucagon (Eli Lilly & Co., Lot No. 258-234B-93) was used. This preparation contained no detectable amount of insulin when measured by an immunochemical assay described previously(5). Glucagon-$I^{131}$ was prepared by a method used for labeling of insulin-$I^{131}$(8). Specific activity of the preparations varied from 2.5 to 12.0 mc/mg.

Two of 4 male albino rabbits, weighing 2.5-3.0 kg, were immunized with glucagon suspended in either Bayol-Arlacel or Freund's adjuvants, as described by Hayashida and Li(9). The other 2 animals were immunized with glucagon conjugated to ovalbumin with bis-diazo benzidine(10), similarly suspended in the adjuvants. All 4 animals were given subcutaneous injection of the hormone in doses of 3-10 mg at varying intervals over a 2-year period. Serum samples were obtained 2-3 weeks after each series of injections, frozen and stored.

Titers for glucagon antibodies in serum were determined by a modification of the method previously employed for measuring insulin antibodies(5). Glucagon-$I^{131}$, 0.02 $\mu$g, was incubated for 1 hr at room temperature with 0.2 ml of undiluted serum or of serum serially diluted with 5% human serum albumin. Aliquots, 0.1 ml, were subjected to hydrodynamic flow on paper in veronal buffer, pH 8.6, for 1 hr at room temperature. In this system free glucagon remains at the origin, while antibody-bound glucagon travels as a single band with the serum proteins (11). After the papers had been dried and

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*Supported by grants from U.S.P.H.S. and from Eli Lilly & Co. Cost of special equipment was defrayed in part by a special grant from Smith, Kline & French Laboratories.

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cut into 1 cm segments, distribution of $I^{131}$-activity was determined by counting the strips in a well-type scintillation counter. Total activity in the bound fraction divided by total activity in the free fraction (B/F ratio) was plotted against the dilution of the serum employed. The dilution at which 50% of the chromatographically intact glucagon was effectively bound was taken as the titer value. Chromatographically intact glucagon-$I^{131}$ was taken as the per cent of the glucagon-$I^{131}$ preparation which remained at the origin (hydrodynamic flow) after incubation with pooled normal rabbit serum. For the various glucagon-$I^{131}$ preparations used, this value routinely was 85-90% of the total label.

The influence of immunization on rate of clearance of exogenously administered glucagon was studied in the surviving animal, one of the pair immunized with glucagon conjugated to ovalbumin. Glucagon-$I^{131}$, 4 μg in 2 ml of saline, was injected into the rabbit's marginal ear vein. Serum was then collected at intervals during a 6-hr period for determination of radioactive content. Four control rabbits of equal age were similarly treated. To allow comparison of results between animals of different weight, the percentage of injected dose in the circulation was calculated as follows:

$$\% \text{ of injected dose} = \frac{\text{Activity/ml serum} \times 0.03\ddagger \times \text{body wt (g)}}{\text{Total injected activity}} \times 100.$$

Since both glucagon-$I^{131}$ bound to antibody and degraded glucagon-$I^{131}$ move from the origin during hydrodynamic flow at pH 8.6, these two states of the hormone are not differentiated in serum containing circulating glucagon antibodies. Binding of glucagon-$I^{131}$ to antibody, however, is pH dependent (Fig. 1); it does not occur below pH 4 and reaches a maximum at pH 8-8.5. Degradation products of glucagon-$I^{131}$ in both immune and normal sera were therefore quantitated by adjusting serum pH to 2.5 with 1 N HCl and subjecting an aliquot to hydrodynamic flow in 0.3 N acetic acid. Thus degradation could be determined in sera containing relatively large amounts of antibody. No difference in percentage of degradation of glucagon-$I^{131}$ was found in preparations of normal sera subjected to flow at pH 2.5 and at pH 8.6. The preferential salt precipitation technic used previously for immunochemical assay of insulin(5) was applied to glucagon with one modification: precipitation with sodium sulfate at 25°C, which produces a more compact, more easily centrifuged precipitate, was substituted for precipitation by sodium sulfite at 4°C.

*Results. Development of glucagon antibodies.* None of the sera from untreated animals bound the chromatographically intact glucagon. The development of glucagon antibody titers in rabbits given multiple injections of antigen during the 22-month period is shown in Fig. 2. In all 4 animals, antibody was detectable within 1 month. Two and one-half months later, after the animals had received 3 more injections, the titer of the sera was increased in 3 of the 4 rabbits. At the end of a 5-month rest period all titers had fallen, but they rose significantly again in the surviving rabbits following 2 booster injections. During a second rest period of 9 months, the titers again dropped. At 18 months, the animals still responded to antigen by producing antibody. By 22 months, however, the surviving animals were comparatively unresponsive to the same antigenic stimulus.

*Characterization of antisera.* None of the sera tested bound insulin-$I^{131}$, suggesting that the antisera to glucagon did not contain any detectable amount of antibodies to insulin. Although precipitin reactions did not occur when varying amounts of free glucagon were added to the antiserum from each of the rabbits immunized with the glucagon-diazo-ovalbumin, strong precipitin reactions were readily obtained when glucagon-diazo-ovalbumin itself was added. Preincubation of glucagon with large excesses of antisera did not inhibit the hyperglycemic activity of the hormone as determined by injecting the mixture into normal, fasted cats.

*Effect of antibodies on clearance of glucagon-$I^{131}$.* Three minutes after injection of

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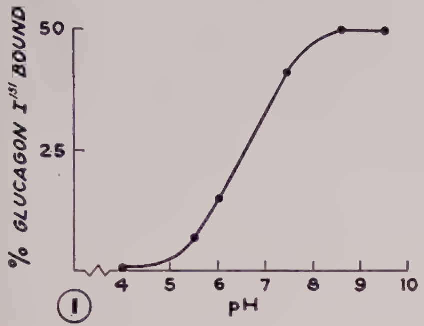
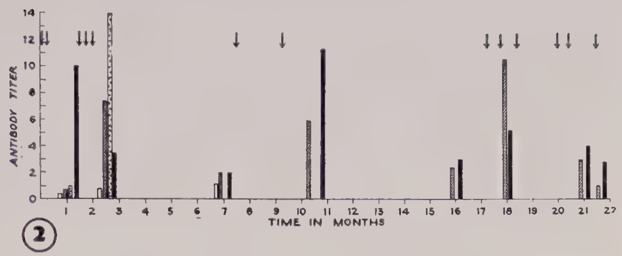
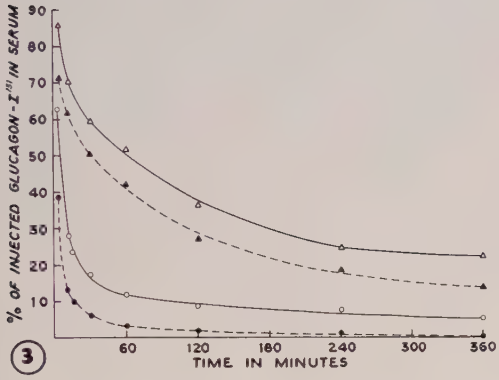
‡ Based on serum volume estimated as 3.0% body weight.







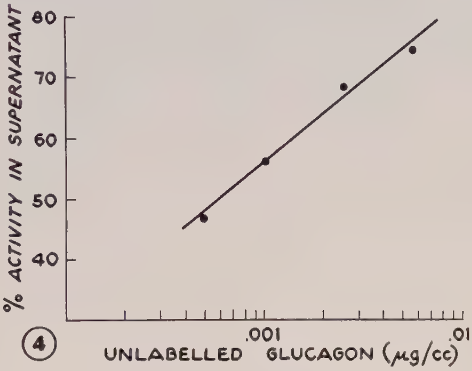


FIG. 1. Effect of pH on binding of glucagon-$I^{131}$ by rabbit antisera. Buffer systems and experimental technic as described by Grodsky *et al.*(8).

FIG. 2. Development of antiglucagon titers in rabbits after intermittent inj. of glucagon preparations suspended in adjuvants. Open bar, striped bar = free glucagon in adjuvants; dotted bar, solid bar = glucagon conjugated to ovalbumin with bis-diazo benzidine and suspended in adjuvants. Arrows indicate inj. of antigen.

FIG. 3. Radioactivity of serum of immunized and normal rabbits after inj. of glucagon-$I^{131}$. △—△ = immunized rabbit, total serum radioactivity. ▲---▲ = immunized rabbit, serum radioactivity representing chromatographically intact glucagon. Values represent mean of 2 experiments. ○—○ = 4 normal rabbits, total serum radioactivity. ●---● = 4 normal rabbits, serum radioactivity representing chromatographically intact glucagon. Values represent mean of 2 experiments on each of the 4 animals.

FIG. 4. Effect of concentration of unlabeled glucagon on binding of glucagon-$I^{131}$ to antibodies obtained from immunized rabbit.

glucagon-$I^{131}$ into an immunized rabbit, 85% of the label was present in the serum (Fig. 3). As determined by hydrodynamic flow at pH 8.6, 82-95% of the glucagon left the origin as either bound or degraded hormone. Examination of the serum at pH 2.5 disclosed that only 15% of this glucagon was degraded. The glucagon remained comparatively intact and was only slowly cleared from the circulation in the subsequent 6 hr. In the normal rabbits, binding of glucagon could not be demonstrated. Both clearance and degradation of glucagon were rapid; at 1 hr, only 3% of the chromatographically intact hormone remained, compared to 42% in the immunized animal.

*Immunoassay for glucagon.* When glucagon-$I^{131}$ and sufficient antisera to bind 50% of the label were incubated in glycine-albumin buffer(5) with increasing concentrations of unlabeled glucagon, the unlabeled glucagon displaced the labeled hormone from the antibody. After precipitation of the bound glucagon with $Na_2SO_4$ through the range 0.0005-0.005 μg of glucagon per ml, the per cent activity displaced into the supernatant was proportional to the log of the glucagon concentration (Fig. 4). Therefore, the preferential salt precipitation technic previously reported for immunoassay of insulin appears adaptable for detecting small amounts of glucagon.

*Discussion.* Our observations and those of Unger *et al.*(7) leave little doubt that glucagon, when injected into rabbits in suitable adjuvant, produces specific antibodies which bind but do not precipitate glucagon. In our study, measurable antibody appeared in the serum within one month of injection of either glucagon or conjugated glucagon as antigen. Subsequently, the level of antibody increased progressively after repeated antigen injections. After resting periods of 5-9 months, antibody titers declined as expected, but a significant level of circulating antibody was still present in each animal.

The dissociation of glucagon from its antibody *in vitro* with decreasing pH followed a curve almost identical to that previously reported for insulin and its antibody(8) and made possible the development of a technic for measuring both degraded glucagon and immunochemically bound glucagon in aliquots of the same serum. The marked retention of glucagon-$I^{131}$ bound to antibody in the circulation of immunized rabbits was similar to that reported in similar insulin studies(12). The sensitivity of the antibody-bound glucagon to "glucagonase" activity in the liver may be decreased, since degradation products appeared far more slowly in the serum of the immunized rabbit than in sera of the control animals. It is noteworthy that the inhibited clearance of insulin and glucagon in presence of their respective antibodies is the exact reverse of the phenomenon described for most other antigen-antibody systems(13), namely, increased clearance of the complex. Both insulin and glucagon are proteins of low molecular weight and are not precipitated with their specific antisera. Possibly such soluble complexes are removed by the liver through a different mechanism than that required for clearance of precipitating antigen-antibody complexes.

The application of the preferential salt precipitation technic makes possible the use of glucagon-$I^{131}$ of low specific activity for immunochemical assay of extremely small amounts of glucagon.

*Summary.* The antibody content of sera from rabbits treated intermittently with free glucagon or glucagon conjugated with ovalbumin was measured at intervals during a 2-year period. Circulating nonprecipitating antibody, capable of binding glucagon-$I^{131}$, was detectable within one month, and was still detectable after 4 to 5 month periods during which no antigen was administered. Glucagon-$I^{131}$ injected into an immunized rabbit was rapidly bound to antibody, remained chromatographically intact and was only slowly cleared from the circulation. Glucagon-$I^{131}$ could be dissociated from its antibody at acid pH. The preferential salt precipitation technic used for immunochemical assay of insulin proved applicable to measurement of minute amounts of glucagon.

The authors are indebted to Dr. Otto K. Behrens, Eli Lilly & Co. for supplies of recrystallized glucagon and for tests of antibody inhibition of glucagon activity in cats. We wish to acknowledge the technical assistance of Miss Angela Lee.

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Received January 16, 1961. P.S.E.B.M., 1961, v107.

# Lipophilic Colloids of Yttrium-90: Preparation and Tissue Localization in Animals Following I.V. Administration. (26666)

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Essentially all radiocolloids which have been used in palliation and treatment of leukemia and other malignancies have been inorganic and hydrophilic in nature. Such materials (*e.g.* Au-198, chromic phosphate-P-32, yttrium phosphate-Y-90, etc.) are generally accumulated in reticuloendothelial tissues of the body following *i.v.* injection, presumably by phagocytosis. Thus, liver, spleen, bone marrow, and lymph nodes are principal sites of colloid deposition; lung, kidney, and adrenal receive intermediate amounts of activity; while blood, muscle, testis, ovary, brain and other tissues are essentially devoid of radioactivity. In addition to this typical tissue distribution pattern, some radiocolloids may partially dissociate within the body and the resultant "ionic" dissociation products be deposited in bone.

The particle size of the colloid micelle has sometimes been regarded as the property most important in determining the site of tissue localization. Hahn(1) however, recognized the importance of surface coating and contrasted the distributional behavior of gold colloids and silver-coated gold colloids. Lahr (2) also observed this phenomenon and reported different tissue depositions for colloids coated with certain organic materials. In the light of these reports, it was felt that a lipophilic colloid might demonstrate a unique distributional pattern and might, therefore, have unusual therapeutic properties.

Yttrium-90 has been injected *i.v.* with a variety of organic chelating agents(3,4,5). These products were all quite soluble, and their molecular dimensions such that they could hardly be classified as colloidal aggregates. In this investigation, well-dispersed lipophilic colloids have been prepared from yttrium-90 by chelation with (1) oleoylacetone and (2) dodecyliminodiacetic acid. Their tissue localization and excretion have been evaluated in experimental animals.

*Materials and methods. Yttrium-Oleoylacetone colloid.* Oleoylacetone [$CH_3$-$(CH_2)_7$-CH=CH-$(CH_2)_7$-CO-$CH_2$-CO-$CH_3$, Dow Chemical Co.] chelated yttrium and formed a lipophilic emulsion as follows: One ml of 0.02 M $YCl_3$ was added to 4.5 ml of an acetone solution containing 0.46 ml of oleoylacetone. This clear solution was then mixed with 10 ml of a solution containing 10% glycerine and 0.3% soluble egg lecithin in distilled water. The turbid emulsion was boiled 5 minutes to expel acetone and allowed to cool. The pH was adjusted to phenolphthalein end-point with 1 N. NaOH. The final product was autoclaved 15 minutes and stored at 5°C.

*Yttrium-dodecyliminodiacetic acid colloid.* Dodecyliminodiacetic acid (D.I.A.) was synthesized according to Stein(6). Seven ml of 0.02 M. D.I.A. was adjusted to phenolphthalein end-point with NaOH. To this was added dropwise 1.0 ml of 0.02 M. $YCl_3$. Dilute NaOH was added simultaneously with stirring to maintain alkalinity. To assure isotonicity, 3 ml of 2.3% NaCl was also added. The resultant product was pasteurized at 65°C for one hour and stored at 5°C.

*Yttrium-90 colloids.* The radioactive yttrium (1 to 25 mc of $YCl_3$ solution, Oak Ridge Nat. Labs., Union Carbide Nuclear Co.) was equilibrated 15 minutes at room temperature with 1.0 ml of 0.02 M $YCl_3$ carrier solution and employed for preparation of colloids exactly as described above.

*Assay procedures, dialysis technic, animal toxicity and tissue localization methods.* As previously employed in these laboratories (7).

*Results. Properties.* Both products were well-dispersed colloids which showed no flocculation or sedimentation after prolonged storage at 5°C. Neither seemed pyrogenic to the animals tested, nor caused hemolysis when mixed with whole citrated human blood.

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* From thesis submitted in partial fulfillment of requirements for M.S. degree, Baylor Univ. Graduate Research Inst.

The yttrium-oleoylacetone reaction product, after dispersion in water, formed a slightly turbid, pale yellow emulsion. Microscopic examination revealed a very few large aggregates ($>1$ $\mu$), but the majority were less than 0.2 $\mu$ in diameter. By contrast, Y-D.I.A. formed an almost completely clear dispersion with minimal Tyndall effect. Individual micelles were not visible by ordinary light microscopy.

To confirm the complete conversion of ionic yttrium to a colloidal product, both chelates were dialyzed against a large excess of saline at pH of 8.0. No $Y(OH)_3$ precipitated during this process. The yttrium-oleoylacetone sol showed 0.05 to 3.0% dialyzable radioactivity, the Y-D.I.A. 3.5 to 5.5%. This tends to substantiate that a complex has been formed by each chelating agent. Such a conclusion also seemed warranted by other observations made during this investigation. Thus, as dilute sodium hydroxide was added to yttrium chloride solution, a heavy, white precipitate of $Y(OH)_3$ formed *in the absence of a suitable complexing agent.* When, however, a potent complexing agent was present, as with oleoylacetone and n-dodecyliminodiacetic acid, no such precipitation occurred. Such behavior strongly suggests that a complex has been formed, and that its dissociation constant is less than the very low solubility product constant of $Y(OH)_3$ at this pH.

It was found that the Y-D.I.A. was precipitated by addition of an equal volume of absolute methanol. The solubility characteristics of this material in water, methanol, and 50% methanol showed clearly that it was neither $Y(OH)_3$ nor n-dodecyliminodiacetic acid. Further, when dried under nitrogen, weighed, and ignited to constant weight, its ash content (presumably $Y_2O_3$) corresponded closely to a 1:1 molar ratio of reactants. Preliminary evidence suggests that yttrium and oleoylacetone have combined in a 1:3 molar ratio in this product.

TABLE I. Tissue Deposition of Y-90 in Rats after I.V. Oleoylacetone-Y-90 Chelate.

| Tissue | Relative specific activity* | |
|---|---|---|
| | Day 1 | Days 2–8 |
| Liver | 12.3 | 11.9 |
| Spleen | 9.1 | 13.8 |
| Marrow | 5.3 | 4.4 |
| Bone | 4.9 | 4.9 |
| Lung | 2.7 | .8 |
| Kidney | 3.9 | 2.5 |
| Blood | .30 | .12 |
| Muscle | .06 | .02 |
| Skin | .07 | .05 |
| Brain | .02 | .01 |

* Calculated on basis of recovered activity.

*Tissue deposition of yttrium-oleoylacetone.* Tissue localization of yttrium-oleoylacetone following *i.v.* administration to albino rats (8 animals) is presented in Table I. Results are expressed in Relative Specific Activity (R.S.A.), *i.e.,* counts per minute per gram of tissue under consideration divided by total counts per minute per gram of animal as a whole. Suitable corrections have, of course, been introduced to account for radioisotope decay. Results so reported seem to facilitate comparisons between animals and between species, independent of dose injected and organ size. With this colloid, despite its lipophilic character, tissue distribution involved primarily the R.E. system in a manner quite analogous to the more common, hydrophilic radiocolloids. Some enhanced lung uptake was apparent; perhaps a few larger than average aggregates have been trapped in the capillary bed as suggested by the work of Gabrieli(8).

Following *i.v.* administration, carrier-free yttrium salts are deposited primarily in bone and show an accelerated urinary excretion (9,12). With the quantities employed in these experiments (15 $\mu$g/rat), one might anticipate such results if the chelate were rapidly dissociated *in vivo.* Indeed, some enhanced bone and kidney deposition of yttrium suggests partial metabolic dissociation of this chelate. Presumably, the resultant "ionic" dissociation products would demonstrate a tissue deposition pattern similar to that of carrier-free yttrium salts.

*Tissue localization of yttrium-D.I.A. colloid.* Results, expressed as R.S.A., are presented in Table II for 8 FBW mice and in

TABLE II. Tissue Deposition of Y-90 in Mice after I.V. Injection of Y-D.I.A.

| Tissue | Relative specific activity* Day 1 | Day 3 | Day 7 |
|---|---|---|---|
| Liver | 9.8 | 14.0 | 14.2 |
| Spleen | 9.7 | 18.9 | 37.0 |
| Bone | .73 | 1.0 | 1.1 |
| Lung | .87 | 1.0 | 1.3 |
| Kidney | .91 | .71 | .69 |
| Blood | .06 | .01 | .003 |
| Muscle | .06 | .03 | .03 |
| Skin | .15 | .09 | .10 |

* Calculated on basis of injected activity.

Table III for 2 mongrel dogs following *i.v.* injection of Y-D.I.A. These R.S.A. values are based on total injected activity. As before, this lipophilic colloid gave essentially a typical R.E. system distribution, but with less uptake by lung, bone, and kidney than noted above. The slow blood clearance of colloid in dogs was unexpected and somewhat at variance with previous experience in this institution.

In mice the increase in R.S.A. of spleen after the first day does not represent an actual increase in total amount of activity in this organ. This change appears to arise from a decrease in weight of the spleen. Thus, average spleen weight decreased as a function of time after colloid injection (Table IV), and one must conclude that the "tracer" doses employed in this experiment (10 $\mu$c/20 g mouse) caused some radiation injury. In light of our own toxicity experiments and the experience of others, it seems most unlikely that chemical or pharmacologic effects of non-radioactive yttrium could produce such a result.

Combined excreta from 3 mice were assayed to evaluate excretion rates. Excretion of Y-90 decreased from 5.6%/day to 3.1%/day during a 7-day interval following injection.

TABLE III. Tissue Localization of Y-90 in Dogs after I.V. Injection of Y-D.I.A.

| Tissue | Relative specific activity* | Tissue | Relative specific activity* |
|---|---|---|---|
| Liver | 8.3 | Lung | 0.7 |
| Spleen | 12.2 | Kidney | 2.1 |
| Marrow | 5.0 | Thyroid | 0.4 |
| Blood | 0.6 | Ovary | 1.2 |
| Bone | 0.9 | Muscle | 0.05 |

* Mean for 2 animals sacrificed at 2 days. Calculated on basis of injected activity.

Six mice received 0.1 ml of Y-D.I.A. (10 $\mu$c) *i.p.* Upon sacrifice at 3 and 7 days, these animals showed good localization of Y-90 within the peritoneal cavity. Of the little which left the cavity, most was accumulated within bone. Mean R.S.A. = 0.37.

*Discussion.* In Table V is summarized the tissue distribution of lipophilic colloids of this investigation together with the hydrophilic colloids previously studied in this Institute (7,10,11). All previous data have been recalculated and results expressed in R.S.A. to facilitate comparisons. Since blood R.S.A. decreased with time in all cases, values of less than 24 hours post-injection are not included. Averages for other organs include data from one hour to several weeks post-injection, for there appears little redistribution of activity with time. R.S.A. values for spleen in the 7-day mice were corrected for organ weight loss in the case of Y-D.I.A. All averages were taken from R.S.A. values based on total recovered activity. Many tissues having very low R.S.A. have been omitted for sake of clarity.

TABLE IV. Splenic Atrophy Following 0.5 $\mu$c/g Y-D.I.A. in Mice.

| Days after inj. | Spleen wt, g* |
|---|---|
| 1 | .0793 |
| 3 | .0535 |
| 7 | .0181 |

* Mean for 3 mice/group.

Though the number of individuals included in each mean value is small and the variance between individuals considerable, some observations appear warranted: (1) All radiocolloids were accumulated readily by liver and spleen in all species considered, though this effect is less marked with $YPO_4$ and $Y(OH)_3$. (2) Lung and kidney show low R.S.A. except for glucose-suspended zirconyl phosphate in man. The enhanced kidney concentration in this case may reflect kidney damage and leukemic infiltration in these terminal patients, though an accelerated excretion of radioactivity cannot be excluded. (3) R.S.A. of bone marrow is probably lower than one would desire for radiation therapy

TABLE V. Comparison of Tissue Distributional Behavior of Certain Radiocolloids after *I.V.* Administration.

| Radiocolloid and recipient | Relative specific activity* | | | | | | | Ref. |
|---|---|---|---|---|---|---|---|---|
| | Liver | Spleen | Lung | Kidney | Marrow | Blood | Bone | |
| Chromic phosphate (in Kelcosol) | | | | | | | | |
| Rat | 22.2 | 18.2 | 1.3 | .57 | 2.6 | .08 | .69 | (11) |
| Man | 13.0 | 13.2 | 3.3 | .82 | 2.8 | — | — | (11) |
| Zirconyl phosphate (in dextran) | | | | | | | | |
| Rat | 19.0 | 146.0 | 1.5 | 1.4 | — | .32 | — | (7) |
| Dog | 27.3 | 11.6 | .92 | 1.1 | 2.4 | .75 | — | (7) |
| Man | 9.7 | 14.4 | 2.2 | 2.9 | 3.7 | .28 | .14 | (7) |
| Zirconyl phosphate (in glucose) | | | | | | | | |
| Man | 9.1 | 8.0 | 2.0 | 8.6 | 2.6 | .8 | — | (7) |
| Yttrium hydroxide (in maltose) | | | | | | | | |
| Mouse | 6.8 | 3.6 | .53 | 1.6 | — | .13 | 6.6 | (10) |
| Man | 2.7 | 6.2 | .52 | 1.5 | 10.5 | — | 3.5 | (10) |
| Yttrium phosphate (in carboxy-methyl-cellulose) | | | | | | | | |
| Mouse | 4.1 | 1.4 | .26 | .81 | — | .16 | 10.0 | (10) |
| Man | 5.9 | 7.2 | 3.6 | 1.1 | 4.4 | — | 3.0 | (10) |
| Yttrium oleoylacetone (Aq. glycerol and lecithin) | | | | | | | | |
| Rat | 12.1 | 11.4 | 1.9 | 3.2 | 4.8 | .29 | 4.9 | |
| Yttrium dodecyliminodiacetic acid | | | | | | | | |
| Mouse | 18.2 | 16.2 | 1.4 | 1.0 | — | .03 | 1.3 | |

* Calculated on basis of recovered activity.

of chronic leukemia and related blood dyscrasias, except for yttrium hydroxide, (4) bone accumulation of radioactivity very probably reflects partial metabolic dissociation of the colloids followed by redistribution of "ionic" dissociation products. This is certainly suggested by the data for yttrium hydroxide, phosphate and oleoylacetone chelate. (5) The lipophilic colloids of yttrium show essentially the same tissue distribution pattern as the inorganic, hydrophilic colloids. Lipophilic character would not, therefore, seem to be a major determinant of tissue deposition of radiocolloids.

*Summary.* Two new lipophilic colloids of radioyttrium, Yttrium-Oleoylacetone Chelate-Y-90 and Yttrium-Dodecyliminodiacetic Acid Chelate-Y-90, have been prepared. Their tissue deposition in experimental animals following *i.v.* administration has been shown to compare closely with that of the more common inorganic, hydrophilic radiocolloids. Lipophilic character appeared to confer on these products no unique tissue distributional behavior.

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Received January 19, 1961. P.S.E.B.M., 1961, v107.

# Studies on Serum Haptoglobin in Experimental Connective Tissue Disorders.*† (26667)

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Haptoglobin (Hp)¶ is a serum glycoprotein with the mobility of an alpha-2 globulin characterized by its property of combining irreversibly with hemoglobin(1,2). High serum haptoglobin levels have been found in human subjects in many different pathologic conditions(3,4). Hp values are particularly high in diseases with connective tissue involvement, *e.g.*, rheumatic disease, systemic lupus erythematosus, metastatic cancer. Study of serum Hp in experimental animals and experimental production of hyperhaptoglobinemia are necessary steps to investigate the mechanism of normal and pathological Hp production. The purpose of the present report is to show that different experimentally produced connective tissue disorders in guinea pigs and rabbits are accompanied by very high serum Hp levels. It has been shown that high serum Hp levels occur during development of carrageenan granuloma in rats and guinea pigs(5) and following turpentine injection(6). These findings are now confirmed and extended.

*Methods and materials.* Guinea pigs were used for induction of carrageenan granulomas(5) and acute scurvy. Rabbits were used for amyloidosis production by biweekly subcutaneous turpentine injections, and for the study of Hp in sera of animals after a single intravenous injection of different enzymes. Enzymes used and their dosage were: Papain (Nutritional Biochemicals, 14 mg/kg); bovine testicular hyaluronidase (Armour, 3500 USP units-110 mg/kg); trypsin (Armour, cryst., 3530 units-1 mg/kg); chymotrypsin (Armour, cryst., 14000 Armour units-12 mg/kg); elastase (prepared by a modified Banga method(7) from porcine pancreatin, Armour, having a specific activity of 11.4 azoelastase units/mg protein(8), injected at a concentration of 18 mg protein/ml, 1 to 3 ml). Control animals received the same volume of sterile saline, or 1.5 mg of ovalbumin in 1.5 ml. In the scurvy experiments two types of controls were used: animals fed the scorbutogenic diet *ad libitum,* and pair-fed animals, both groups receiving a 2.5 mg supplement of ascorbic acid daily by mouth. The experimental animals received the scorbutogenic diet without Vit. C supplement. Serum samples were stored frozen until used for determinations. Hp was determined by a colorimetric modification of Jayle's "activation" method(9) using ethylperoxide, 0.05 N., as the substrate and guaiacol, Merck, dissolved to 5 ml/l in 0.1M acetate buffer at pH 4.2 as the hydrogen donor. Ten and 20 $\mu$l of each serum were mixed with 0.1 ml of beef cyanmethemoglobin, recrystallized once according to Drabkin(10), in a solution of 0.05 g/l. The volume was made up to 0.2 ml with saline, 5 ml of the guaiacol solution were added and finally 1 ml of the peroxide solution. The color developing in exactly 6 minutes at 30°C was read in a Coleman Jr. Spectrophotometer at 470 m$\mu$ in a 13 × 13 mm rectangular cuvette. A saturation curve, prepared with high titer guinea pig and rabbit serums, was used to establish the optical density corresponding to the peroxidase activity of the saturated hemoglobin-haptoglobin complex. Using this value, the 6-minute optical density tracings were converted to hemoglobin binding capacity (HBC) and expressed in mg hemoglobin bound to 100 ml of serum.

*Results.* Table I summarizes results of the Hp determinations. Average values were

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* Supported by grants from U. S. Public Health Service and Am. Cancer Soc.

† The authors wish to thank Prof. M. F. Jayle and Dr. Anette Marnay for a gift of ethylperoxide.

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¶ Abbreviations used: Hp - haptoglobin; HBC - hemoglobin binding capacity

TABLE I. Serum Haptoglobin Values (Expressed as Hemoglobin Binding Capacity, HBC) of Guinea Pigs and Rabbits Subjected to Various Treatments.

| Animal | No. | Treatment | HBC, mg % serum | t* |
|---|---|---|---|---|
| Guinea pigs | 16 | Normal controls | 10.7 ± 2.3 | — |
| | 7 | Pair-fed | 27.4 ± 5.9 | 2.95 |
| | 19 | Carrageenan granuloma | 38.1 ± 6.1 | 4.5 |
| | 14 | Acute scurvy | 82.6 ± 12.8 | 5.9 |
| | 7 | Scurvy and carrageenan | 53.0 ± 7.0 | 5.64 |
| Rabbits | 58 | Normal controls | 70.0 ± 3.8 | — |
| | 10 | Turpentine inj.; after 3 mo | 182.7 ± 15.8 | 5.0 |
| | 5 | Same, after 8 mo | 118.0 ± 39.5 | 1.1 |
| | 11 | Papain I.V. | 165.3 ± 13.5 | 4.7 |
| | 5 | Elastase I.V. | 236.0 ± 76.0 | 2.17 |
| | 9 | Hyaluronidase I.V. | 139.0 ± 17.4 | 3.9 |
| | 3 | Trypsin I.V. | 98.1 ± 10.0 | 1.5 |
| | 3 | Chymotrypsin I.V. | 53.0 ± 13.0 | 0.9 |
| | 3 | Ovalbumin I.V. | 100.0 ± 8.6 | 1.7 |
| | 4 | Saline I.V. | 114.2 ± 21.5 | 1.6 |

* 95% significance if over 1.9 and 99% significance if over 2.6.

calculated as follows: For guinea pigs the determinations were done for normal controls and for pair-fed animals at the same time as for animals maintained on a vitamin-free diet. The acute scurvy group was bled 3 weeks after the scorbutogenic regimen was started; the carrageenan injected animals were bled on the 8th day after injection which corresponded to the 21st day of the different dietary regimens. Hp values obtained on these samples were then respectively averaged (4th column of Table I).

In the rabbit experiments, blood was taken before treatment and at different intervals after injection. Normal control values were averaged without respect to the time at which blood was taken. For the turpentine treated group the 3-month and 7-month values are given: at 3 months no amyloid was detectable, while at 7 months (and generally after the 4th month) all the animals had amyloid deposits in the spleen and less severe involvement of liver, kidneys and other tissues. Animals receiving intravenous enzyme, protein or saline injections were bled before injection and at 9, 18, 50 and 96 hours after the injections. The results presented are the average of the highest values of these time curves, without respect to the time when this value was found. These peak values generally appeared between 9 and 50 hours after injection (Fig. 1). The significance of the difference was calculated between the mean Hp value established as indicated and the mean of the corresponding normal group (11).

The guinea pigs bearing carrageenan granulomas have a nearly 4-fold mean Hp than the normals, a result similar to that obtained previously using Jayle's iodometric Hp determination(5). Acute scurvy is accompanied by very high serum Hp levels, some 8 times the normal average. It is interesting to note the somewhat lower average HBC in scorbutic guinea pigs bearing granulomas as compared with the simple scurvy group. However, this difference is not significant at the 95% level. The mean HBC of the pair-fed groups was significantly higher than that of the normal controls. The reason for this

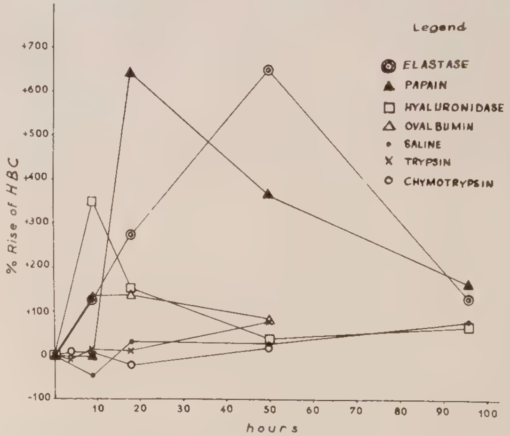

FIG. 1. Serum haptoglobin (HBC) as a function of time after intrav. inj. into rabbits of different enzymes, proteins and saline.

increase is not apparent although the pair-fed animals lost some weight during the experiment.

Repeated turpentine injections in the rabbit resulted in generalized amyloidosis. At 3 months after start of injections serum Hp is about 2.5 times above the normal level, then falls off when amyloid begins to be detectable in the spleen. Seven months after start of treatment, when amyloidosis is widespread, Hp is still higher than the normal average but the difference is no longer significant. Intravenous papain injections in rabbits according to the technic of Thomas (12) results in a spectacular rise of serum Hp. Similar results were obtained with intravenous elastase and to a somewhat lesser extent with hyaluronidase injections. Eighteen to 50 hours after papain or elastase injections, serum Hp can be as high as 7-fold the initial value. Fig. 1 gives the time course of serum Hp level in some typical experiments. Though a rise can sometimes be obtained even after saline or ovalbumin injections, the mean highest values of these groups are not significantly different from the average of the normal control group. The same holds for trypsin and chymotrypsin injections. Following reinjection of papain at 100 hours, HBC levels were again elevated to values comparable with those obtained after the initial injection.

The behavior of acid polysaccharides was investigated in some cases by uronic acid determinations using a modified Tollens method(13). In contradistinction from the effect of papain(14), both hyaluronidase and elastase injections are followed by a decrease of uronic acid in the serum, followed by a return to initial values after 50 hours.

*Discussion.* A striking relation between degradative changes in connective tissue and high levels of circulating protein bound polysaccharide was indicated by Catchpole(15), Engel(16), and Pirani and Catchpole(17). Jayle and Boussier(1) adapted this idea to the relation between Hp production and connective tissue degradation and claimed a mesenchymal origin for this glycoprotein. Though there seems to be no quantitative difficulty for this hypothesis(18) a direct proof is not yet available to sustain it.

The present results show that many different treatments produce a considerable rise of serum Hp levels. Some of the treatments used (scurvy, carrageenan granuloma, turpentine and enzyme injections) are of such a nature that one could reasonably suppose a more or less extensive alteration of connective tissue. This has been demonstrated for scurvy(17) and papain(12,14). The carrageenan granuloma and the turpentine abscess both produce rapidly anabolizing and catabolizing new connective tissue. These results agree with clinical experience(3) that rise of serum Hp in humans may be part of a nonspecific inflammatory reaction. However, diet restriction as in pair-fed animals, where none of the above effects would be expected, also produces a significant rise of the serum Hp. It is interesting to notice that the highest Hp levels found during turpentine injections are attained well before the actual appearance of amyloid. With the deposition of amyloid, Hp levels tend to decrease.

Simultaneous determination of uronic acid and of Hp show that there is no parallelism between serum levels of acid mucopolysaccharides and Hp following intravenous injection of enzymes. Papain was the only enzyme producing a rise of uronic acid-containing polysaccharides. Here too, the rise of these mucopolysaccharides appeared well before the rise of Hp, the former reaching their highest level between 10 and 20 hours after injection, the latter at 30 to 50 hours. The mechanism by which elastase and hyaluronidase lowered serum uronic acid concentration is not evident, though elastase might attack the euglobulin fraction which contains the mucopolysaccharide of serum (19).

Intravenous papain and elastase injections might be used with advantage for an experimental study of the mechanism of biosynthesis and release of Hp.

*Summary.* Serum haptoglobin was studied in guinea pigs and rabbits in normal animals and after experimental production of connective tissue damage such as in acute scurvy, carrageenan granuloma, turpentine-induced

amyloidosis, and intravenous injection of different enzymes (papain, elastase, hyaluronidase, trypsin, chymotrypsin); controls received intravenous ovalbumin and saline. Significant increase of serum haptoglobin was found in acute scurvy (average 8 times normal), carrageenan granuloma (4 times normal) or both combined (5 times normal), and after intravenous injection of papain, elastase and of hyaluronidase (average of 2 to 2.5 times normal). Particularly striking results were obtained with papain and elastase which in some cases produced an increase in haptoglobin up to 7 times the starting value. These methods may be used to obtain reproducible experimental hyperhaptoglobinemia.

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Received February 3, 1961. P.S.E.B.M., 1961, v107.

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# Interrupted Enterohepatic Circulation of Bile and Its Effect on Urinary and Biliary Excretion of $I^{131}$.* (26668)

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Any alteration of hepatic physiology markedly affects iodine metabolism. Carbon tetrachloride-induced liver injury results in both increased hepatic retention and decreased thyroid uptake of subcutaneously administered $I^{131}$(1). Shunting portal circulation by an Eck fistula leads to a decreased thyroid uptake of $I^{131}$ following its intragastric administration(2). Absence of bile in the intestinal tract impairs the absorption of orally administered $I^{131}$(3). It is not known, however, whether bile loss will modify the metabolism of $I^{131}$ intravenously administered.

The purpose of these experiments was to study the effect of interrupted and uninterrupted enterohepatic circulation on the biliary and urinary excretion of iodide administered intravenously. Also, efforts were made to ascertain the conditions necessary to maintain normal urinary iodide excretion after surgery upon the biliary tract and alteration of normal pathways of bile flow.

*Materials and methods.* Nine groups (Table I) of male Holtzman rats weighing approximately 150 g were used. Surgery was performed under Nembutal anesthesia.

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*This investigation was supported by the Research and Development Division, Office of the Surgeon General, Dept. of the Army.

TABLE I. Effect of Total Biliary Diversion on Urinary and Biliary Excretion of $I^{131}$.

| Group | Operation; additional experimental conditions | No. of rats | Urine (excretion/24 hr) % of injected $I^{131}$* | Urine Vol (ml)* | Urine Sodium (meq)*β | Bile (excretion/24 hr) % of injected $I^{131}$* | Bile Vol (ml)* |
|---|---|---|---|---|---|---|---|
| I | Control, no choledochostomy | 14 | 78.3 ± 3.7 | 16.7 ± 2.1 | 1.86 ± .26 (8) | | |
| II | Bile fistula | 6 | 4.9 ± 1.3† | 21.2 ± 4.4 | 0.29 ± .10 (8)† | 9.5 ± .49 | 11.4 ± .5 |
| III | " " , $I^{131}$ inj. 2 hr post-op. | 6 | 18.5 ± 7.8† | 26.5 ± 2.4† | .24 ± .10 (8)† | 9.5 ± 1.02 | 15.1 ± .7‡ |
| IV | Sham choledochostomy; reanastomosed bile duct patent | 7 | 78.3 ± 4.8‡ | 18.7 ± 3.2 | | | |
| V | " " ; " " " obstructed | 4 | 63.1 ± 8.9‡ | 12.7 ± 2.3 | | | |
| VI | Control; fasted during recovery and collection period, no choledochostomy | 16 | 52.6 ± 8.9†‡ | 15.0 ± 1.4 | .42 ± .14 (7)† | | |
| VII | Bile fistula; donor bile introduodenally infused instead of tap water | 7 | 36.2 ±11.4†‡ | 22.5 ± 6.6 | | 9.5 ± 2.05 | 15.7 ± .7‡ |
| VIII | Bile fistula; offered I-drinking water instead of tap water | 8 | 48.2 ± 6.2†‡ | 30.9 ± 9.1 | | 9.0 ± 1.06 | 11.8 ± .8 |
| IX | Bile fistula; donor bile intraduodenally infused, offered I-drinking water | 8 | 75.5 ± 5.3‡ | 40.0 ± 8.1 | | 3.9 ± .53‡ | 16.6 ±1.5‡ |

* Mean ± S.E. † Significance $<5\%$ when compared to Group 1, control. ‡ Significance $<5\%$ when compared to Group II, bile fistula.
β Na excretion determined from similarly treated, but different, samples of rats (figure in parentheses denotes No. of rats used for each group).

The femoral vein was cannulated in all groups including the controls. At the end of the designated recovery period 1 μc of $NaI^{131}$,† carrier free and diluted with 5% glucose, was injected into the femoral cannula. All rats were permitted to recover 18 hours before injection of radioactive iodide except those in Group III, which were injected 2 hours after surgery.

In sham choledochostomy, anastomosis of the severed bile duct (choledochocholedocostomy) was established by means of a polyethylene tube. The anastomosis was judged patent (Group IV) if bile did not appear in quantity in the urine during the experiment and if, at the end of the experiment, bile dripped from the end of the proximal portion of the bile duct after severance of the polyethylene tube. Otherwise, the anastomosis was judged obstructed (Group V).

Another control consisted of rats fasted during the 18 hour recovery period and the 24 hour collection period, but not subjected to choledochostomy (Group VI).

The basic experimental preparation was an externally draining bile fistula, and an intraduodenal infusion of tap water (Group II). The common bile duct was divided and polyethylene tubes (Pe-10, Pharmaseal) were inserted into the cut ends of both proximal and distal portions to permit both measurement of total bile output and infusion of tap water into the duodenum. Variations in this basic experimental preparation are described below and indicated in Table I.

In 2 experiments (Group VII and IX) bile was substituted for tap water in intraduodenal infusions. The bile had been previously collected at room temperature from donor rats. Donor bile or tap water was infused by means of a constant infusion pump at a rate similar to that at which bile drained from the fistula (1 cc/hr). In 2 experiments (Groups VIII and IX) iodinated drinking water (Lugol's solution diluted to 24 mg % iodide) was offered 48 hours prior to choledochostomy and throughout recovery and collection periods instead of tap water. Tap water was continuously available to all other groups; all except the fasted group (VI) were fed Purina Laboratory Chow *ad lib.*

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† Abbott Laboratories, Chicago, Ill.

For 24 hours following injection of $I^{131}$, urine from all groups and bile from those with a bile fistula were collected in 250 ml small mouth bottles to which was added 1 ml of Na OH-Na I solution to prevent loss of $I^{131}$. The volume of each sample was recorded and then diluted to the volume of the radiation standard. Measurement of radioactivity was conducted with a scintillation well counter united to a Nuclear-Chicago Radiation Analyzer-Scaler combination. Iodide-131 excretion was calculated as percent of the injected dose appearing in urine or bile/24 hours. Bile and urine sodium were determined with a Baird flame photometer.

*Results.* Table I demonstrates the effect of interrupted enterohepatic circulation of bile on urinary excretion of sodium and intravenously administered $I^{131}$. Rats normally excrete about 78.3% of injected $I^{131}$ in 24 hours (Group 1, Control). Rats with a bile fistula (Group II) that had drained 18 hours prior to injection of $I^{131}$, however, excreted only 4.9% of the $I^{131}$ in the same period. Also less sodium was excreted by bile fistula animals than by controls. Sodium and $I^{131}$ excretion were also depressed in bile fistula rats that had been injected with $I^{131}$ only 2 hours postoperatively (Group III).

The possibility that the decrease in urinary excretion of $I^{131}$ was due to operative trauma was tested with Group IV. These rats, with reanastomosed bile ducts, excreted $I^{131}$ at the same rate as the controls. Similarly, rats with partial obstruction of the reanastomosed bile ducts (Group V) showed no significant reduction in excretion of $I^{131}$.

Some diminution of urinary excretion of $I^{131}$ occurred in rats fasted throughout the 18 hour recovery period and the 24 hour collection period (Group VI). However, $I^{131}$ excretion in these fasted controls was significantly greater ($p < .05$) than that of the bile fistula rats (Groups II and III). Sodium excreted by fasted controls was not significantly greater than that excreted by bile fistula rats.

Bile fistula rats in which the collected bile was replaced with rat donor bile (Group VII) or which were supplied with iodinated drinking water (Group VIII) or subjected to a combination of both treatments (Group IX) showed a significant increase in the 24 hour urinary excretion of $I^{131}$ as compared to non-treated bile fistula rats (Group II).

An average of 9.5% of injected $I^{131}$ (Table I) was excreted in bile from bile fistula rats (Group II). Essentially the same activity appeared in bile of rats with a bile fistula injected with $I^{131}$ within 2 hours after choledochostomy (Group III), rats infused with bile (Group VII) or offered iodinated drinking water (Group VIII). Rats with a bile fistula but treated with a combination of bile and iodinated drinking water excreted significantly less ($P < .05$) $I^{131}$ in the bile than did untreated bile fistula rats.

*Discussion.* Several methods of investigation have shown that the liver plays an important role in iodide metabolism. Alterations in hepatic function lead to changes in PBI levels(4) and affect the thyroid uptake of $I^{131}$(5). Bile has been implicated in the metabolism of $I^{131}$ by Friedman and Kowalewski(3) who reported that the absence of bile in the intestinal tract markedly suppressed absorption of orally administered $I^{131}$. They were unable to explain this lack of absorption.

The present report demonstrates the influence of total biliary diversion on metabolism of $I^{131}$ administered parenterally. Rats with an externally draining biliary fistula excreted significantly less $I^{131}$ in urine than controls. In view of the findings by Friedman and Kowalewski(3) it was necessary to determine if this reduced $I^{131}$ excretion was related to an absence of iodide absorption through the intestinal wall. This was accomplished by the fasting controls. Although the excretion rate of $I^{131}$ in fasting controls was less ($P < .05$) than non-fasted controls, it was still significantly greater than that of bile fistula rats and comparable to bile fistula rats treated with either bile or iodinated drinking water. It is possible that diminished iodide absorption plays a minor role in the studied effect.

Fasting and trauma suppress sodium excretion to a much greater extent than they suppress iodide excretion. Sodium excretion is reduced through emotional stress(6) and surgical trauma(7). Trauma was dismissed as a factor in urinary excretion of $I^{131}$ by Van Middlesworth and Berry(8) who reported that intestinal injury, starvation, lack of Vit. $A_1$, $B_2$, and $B_6$, or injections of cortisone had little effect on urinary excretion of $I^{131}$.

Bile loss appears to affect metabolism of iodide to a much greater degree than the iodo derivatives, thyroxine, and triiodothyronine. The liver is mainly responsible for deiodination of thyroxine and triiodothyronine(9). However, dogs with a biliary fistula given Lugol's iodine solution orally metabolized and deiodinated triiodothyronine as affectively as controls(10).

Total biliary diversion markedly suppressed urinary excretion of sodium. This effect may be related to loss of electrolytes in bile as the concentration of sodium in bile‡ was similar to that of blood. However, urinary excretion of $I^{131}$ did not parallel that of sodium in these studies. The sodium content of urine from bile fistula or fasted control rats was similar, but urinary $I^{131}$ of fasted rats was greater than that of bile fistula rats. Conservation of sodium in the fasted or bile fistula rat was probably influenced by hormones since salt depletion has been shown to result in an increased 17-ketosteroid secretion(11).

Intact adrenals were necessary for normal urinary excretion of $I^{131}$(12), but cortisone treatment fully restored $I^{131}$ excretion in adrenalectomized rats(12) and mice(13). The main site of corticosteroid inactivation is the liver(14), and chronic external diversion of bile produced histological signs of hyperfunction in the adrenals and thyroid(15). The influence of total biliary diversion on the urinary excretion of $I^{131}$ intravenously administered may be mediated through the adrenals.

*Summary.* Total biliary diversion markedly depressed urinary excretion of $I^{131}$ intravenously administered. The effect was apparent within 2 hr post choledochostomy. Fasting reduced urinary excretion of $I^{131}$ to a minor extent; operative trauma had no effect. Treatment of bile fistula rats with donor bile and/or iodinated drinking water significantly increased $I^{131}$ excretion. $I^{131}$ content of bile was significantly reduced in bile fistula rats only through combined treatment with donor rat bile and iodinated drinking water. $I^{131}$ content in urine paralleled that of sodium under some conditions but not in others.

The author is grateful to Roy Marrero for expert technical assistance in this study.

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Received February 24, 1961. P.S.E.B.M., 1961, v107.

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‡ 139 ± 3 meq/l, Group II.

## Plasma Antithrombin Alterations Following Oral Papain. (26669)

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We have recently found that oral administration of papain* results in therapeutic activity in man of the same character as that resulting from buccal administration(1). Since this therapeutic efficacy was not accompanied by the untoward side-effects sometimes seen with intravenous enzyme therapy, it appeared that the enzyme was probably not absorbed, but that a plasminogen activator, released by the action of papain in the upper gastro-intestinal tract and absorbed from that site, could be implicated. The lack of systemic absorption of papain is further suggested by the fact that the oral mouse $LD_{50}$ of this enzyme is in the neighborhood of 15,000 mg/kg(2).

Roche e Silva, Andrade, and Teixeira(3) have shown that intravenous infusion of proteolytic enzymes into animals results in the appearance of an antithrombin-like component in blood plasma. Niewiarowski and Kowalski confirmed this, using streptokinase and plasmin in the cat(4). The latter authors have described the anticoagulant as a high molecular weight product of fibrinogen digestion, which can be destroyed by further proteolysis(4). On the basis of this and their own work(5), Wallen and Bergstrom postulated that the protein fragments formed during fibrinogenolysis, and which are substrates for thrombin, can act as competitive inhibitors in the proteolytic reaction of thrombin on fibrinogen, thus contributing to antithrombin activity.

With this background, the present study has been carried out to ascertain whether or not oral papain administration can produce *in vitro* changes which will signal its activity.

This work does not purport to show that oral papain alters *in vivo* clotting mechanism. In fact, in clinical application, no signs referable to changes in clotting mechanism have been observed with oral papain.

*Methods.* Fifteen hospitalized patients who were recovering from recent fractures served as subjects. Blood specimens were collected with 0.4 ml of 5% potassium oxalate for 5 ml of blood.

For assay of plasma antithrombin, the method of Fletcher, Alkjaersig, and Sherry (6) was employed. This test measures clotting time of a patient's plasma following *in vitro* addition of a standardized thrombin† solution. Euglobulin clot lysis determinations were made according to von Kaulla and Schultz(7).

As a low dose of papain, 10 mg were administered every 4 hours by mouth for 48 hours. As a high dose, 200 mg were administered every hour for 4 hours to the same patients. One control clotting time estimation served for both administrations in each patient. Papain was administered as 10 mg uncoated tablets, since enteric coated papain has been previously found to be clinically relatively ineffective(1).

*Results.* Table I clearly demonstrates that the small dose of papain caused a marked increase in plasma antithrombin activity. The high dose resulted in an equally consistent reduction in plasma antithrombin activity. Both sets of results differ from control levels in a statistically highly significant manner. The reduction in plasma thrombin clotting time below that of the control may be explained by the terminal hydrolysis not only of antithrombin formed from fibrinogen but also of the normally circulating antithrombin present in plasma. It is of further interest that patients with reduced thrombin clotting times showed marked acceleration in euglobulin clot lysis time. This finding implies that under the test conditions reduced thrombin clotting time seems to be related to enhanced proteolysis (Table II).

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* Papase—Warner-Chilcott Laboratories.

† Topical Thrombin, Parke-Davis.

TABLE I. Plasma Thrombin Clotting Time (Seconds).

| Reaction mixture: .1 ml plasma + .1 ml thrombin | | | |
|---|---|---|---|
| Patient | Control | After 4 hourly doses of 200 mg each | After 12 doses of 10 mg q.4.h. |
| R.B. | 17 | 10 | 45 |
| D.L. | 16 | 10 | 35 |
| S.K. | 18 | 9 | 35 |
| T.G. | 17 | 12 | 40 |
| R.V. | 17 | 10 | >600 |
| D.L. | 18 | 11 | 24 |
| Y.F. | 17 | 10 | >600 |
| E.C. | 16 | 10 | 27 |
| M.V. | 17 | 9 | 24 |
| S.A. | 17 | 9 | >600 |
| I.D. | 17 | 12 | 30 |
| S.L. | 16 | 11 | 25 |
| R.W. | 19 | 12 | 27 |
| H.J. | 16 | 10 | 26 |
| D.S. | 17 | 10 | 31 |
| Means | 17 | 10 | 25–37* |
| | | p = <.01 | p = <.01† |

* Calculated true mean is between 25–37 at p = .99.

† p = <.01 when values >600 were excluded. p = .02–.05 when values >600 were included. The paradoxical decrease in significance was due to the large increase of stand. error when the >600 figures were added.

TABLE II. Euglobulin Clot Lysis Time after Oral Papain (Hours).

| Dose & frequency | Control | Treated |
|---|---|---|
| 200 mg/hr for 4 hr | 6 | 1 |
| *Idem* | 7 | 3 |
| ” | 9 | 2 |
| ” | 9 | 3 |
| ” | 8 | 2 |
| ” | 6 | 2 |
| ” | 6 | 3.5 |
| ” | 7 | 3 |
| ” | 7 | 2 |
| ” | 6 | 2 |
| ” | 8 | 1 |
| ” | 9 | 3 |
| ” | 8 | 3 |
| ” | 7.9 | 2 |
| ” | 6 | 1 |
| Mean | 7.3 (6.7–7.9)* | 2.2 (1.8–2.6)* |

* 95% confidence limits p = <0.01.

More recent studies appear to show that by increasing the quantity of fibrinogen (plasma) in the thrombin-fibrinogen reaction mixture, a prolonged thrombin clotting time is produced with high papain dosage as well as with low doses. This is compatible with the postulate of Niewiarowski and Kowalski (4) that thrombin inhibition is regulated by concentration of fibrinogen-derived polypeptides in this system.

*Summary.* 1. Administration of small oral doses of papain to human patients results in production of a plasma antithrombin agent demonstrated by prolonged thrombin clotting time *in vitro*. 2. Administration of large oral doses of papain to human patients results in a decrease in thrombin clotting time, and an acceleration of euglobulin clot lysis time. 3. These experiments demonstrate that orally administered papain produces a systemic effect, although in all probability it is not itself absorbed.

We are indebted to Mr. N. Stasilli of the Warner-Lambert Research Inst. for statistical computations.

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Received February 28, 1961. P.S.E.B.M., 1961, v107.

# Effects of Antibiotics on Trachoma Virus in Tissue Cultures.* (26670)

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The propagation of trachoma virus in the yolk sac of the chick embryo by T'ang *et al.* (1) was followed by confirmatory reports (2-7) and by reports of propagation of the virus in explanted tissues from chick embryos(8), and in tissue cells of human origin (9-11). Trachoma virus was identified serologically and morphologically with the psittacosis-LGV family of agents(7,12,13). Propagation of the inclusion blenorrhea agent in the yolk sac of chick embryos substantiated its identification with the same family(14,15).

Propagation of trachoma virus by laboratory procedures provided for screening the sensitivity of virus to drugs(16,17). The screening of drug effect in chick embryos has involved inoculation of drug-virus mixtures simultaneously into the yolk sac of the embryonated egg. Trachoma is an intracellular infection. A response of extracellular virus to drug action may not reflect accurately the response of the intracellular infection in man to such treatment. Jawetz *et al.* (17) attempted to affirm the value of the WHO-recommended treatment with CTC†; however, tetracycline was substituted for CTC and the drug was mixed with the virus prior to inoculation into the yolk sac.

As psittacosis and trachoma viruses replicate and mature in human cells they induce a cytochemical sequence which involves viral DNA, an intermediate RNA matrix around the DNA virus, and emergence of antigenically mature particulate DNA-staining virus (18,19). The cytochemical changes were demonstrable in infected tissue cells stained with acridine orange and observed by ultraviolet microscopy. Antibiotics for psittacosis virus have an ability to interrupt the cytochemically defined maturation of intracellularly established virus(20,21). The technic has now been applied to trachoma-infected cells. This paper is a report of a drug, other than CTC, which has shown therapeutic effect on tissue cells in which trachoma virus was propagating.

*Methods.* The technics concerned with propagation and analysis of psittacosis virus replication have been described(19); the same procedures are applicable to trachoma virus.

*Cells.* Three human cell-lines have been used. (A) The McCoy cell(22) which was originally derived from human synovial tissue. McCoy cells were propagated and maintained in a medium consisting of Mixture 199 plus 0.5% LAH, 5% heat-inactivated calf serum and streptomycin 0.1 mg per ml medium. (B) S 3 clonal strain of HeLa cell (23) obtained from Dr. Harry Eagle, Bethesda, Md. was propagated and maintained in the same medium as for (A) except that 10% heat-inactivated calf serum was used. (C) FL line of human amnion cell(24) obtained through the courtesy of Dr. W. F. McLimons, Atlanta, Ga. The medium for FL cell line was the same as for (A) except that 20% heat-inactivated calf serum was used.

The cells were propagated as monolayer cell-sheets on cover slips in Leighton tubes. The cell sheets were washed twice with Mixture 199 and inoculated with 0.1 ml of trachoma virus in yolk-sac extract or in chorioallantoic fluid. One ml of nutrient fluid was added to each tube and cultures were then incubated at 35°C. At intervals thereafter, the infected cells were stained with acridine orange and examined by ultraviolet microscopy. At intervals after infection, the cell cultures were washed with Mixture 199 and replacement nutrient medium containing antibiotic drugs, in graded dosages, were each added to groups of cultures. The cultures were thereafter examined by fluorochrome microscopy and assayed for virus content by inoculation into fresh tissue cultures(25,11) or into the yolk sacs of 7-day embryonated eggs.

*Virus.* The early trials in tissue cultures were performed with a slow-growing strain

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* Supported by U.S.P.H.S. E2983.

† Chlortetracycline HCl, Lederle Laboratories, Pearl River, N. Y.

of trachoma virus (SA-2) transferred to this laboratory through the generous assistance of Dr. John Snyder of Boston, Mass.(6). More recently, T'ang strain of virus(1) was transferred here through the kind interest of Dr. L. H. Collier of London, England. Both strains of virus were propagated by inoculation of virus in yolk sac extracts into the yolk sacs of 7-day embryonated eggs. Infected eggs usually died within 4 to 7 days and Giemsa-stained smears of their yolk-sacs contained numerous microscopically detected elementary bodies. Stock virus was stored at –50°C until used.

In the course of passaging T'ang virus through alternate yolk sac and tissue cultures (FL cells) a deviant appeared which grew rapidly in the allantoic cavity of embryonated eggs and killed mice rapidly by the intracerebral route. The effects of drugs on both fast- and slow-growing lines of virus were determined.

*Antibiotics.* Six drugs have been tested for effect on trachoma virus propagation in cells: Dihydrostreptomycin $SO_4$; sulfanilamide; penicillin G; chlortetracycline HCl; tylosin tartrate[‡](26,27); and tylosin $SO_4$.* Each drug was diluted in tissue culture medium and added to groups of cell cultures on cover slips. Drug dosages ranged from 200 $\mu$g/ml to 0.5 $\mu$g/ml. The cells were examined at daily intervals thereafter by fluorescence microscopy(19) for evidence of cytotoxicity. Cell cultures on cover slips were inoculated with trachoma virus (as described above) and at intervals thereafter the nutrient fluid was exchanged for nutrient fluid containing a graded dose of one antibiotic. The cells were thereafter examined for cytochemical evidence of virus maturation and washed cell cultures were assayed for infective virus.

*Staining procedures.* Cell cultures with virus were fixed in Carnoy's solution, stained with acridine orange, mounted in buffer pH 3.8 and examined microscopically with ultraviolet light. The technic has been described (19).

*Results.* By the fluorochrome procedure, a pattern of cytochemical changes associated with replicating trachoma virus was visualized which resembled that described for psittacosis virus, except that: (a) the reproductive cycle of SA-2 virus required from 5 to 7 days and the mass of mature virus per cell was relatively small(9); (b) T'ang virus occupied large vacuoles which were 4 to 5 times as large as the viral inclusion; and (c) the fast-growing T'ang virus usually filled the cell with mature virus particles within 48 hours after inoculation. The latter virus killed chick embryos within 3 to 4 days following inoculation into the allantoic cavity and the allantoic fluid from such embryos contained virus. T'ang virus and its enhanced form replicated readily in FL and in McCoy cells but showed less activity in HeLa cells.

Within 24 hours after inoculation of T'ang virus into McCoy and into FL cells, small spheres of RNA-staining material ("red balls") appeared. This represented a matrix of RNA around the initial virus DNA particle (Fig. 1). During the next 48 hours the "red balls" enlarged, multiplied and changed color from red, to orange, to yellow, to green. At the later stage, yellow-green pin-point virus particles appeared (Fig. 2). When various antibiotics were each added to T'ang virus represented by the "red ball" stage a gradient of virus inhibition was observed (Table I). 1. In the presence of 200 $\mu$g of streptomycin per ml nutrient medium the virus attained cytochemical maturity and was infective. 2. Penicillin and sulfanilamide each delayed maturation of virus; however, cultures with each drug contained a significant number of cells with mature virus, and infective virus was recovered from both in fresh cells. 3. CTC interrupted the virus-associated cytochemical sequence at the stage at which it was exposed to the drug. The treated "red ball" appeared solid, homogeneous and intensely red. In a significant number of cells there was no visible evidence of virus by the fifth day of treatment and infective virus could not be recovered from the CTC-treated cultures. 4. Tylosin tartrate, like CTC, interrupted the trachoma virus-induced cytochemical sequence at the stage at which it was added. The "red balls" developed a target-like appearance; the red sphere developed distinctive concentric rings of rarefaction and appeared to be disintegrating. Infective virus

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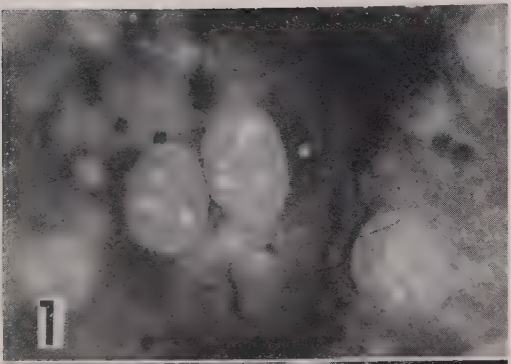
[‡] Eli Lilly and Co., Indianapolis, Ind.



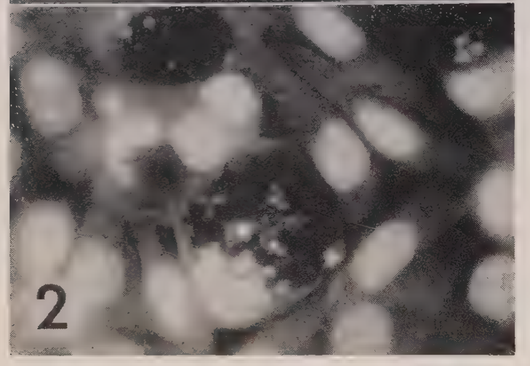


FIG. 1. FL cell infected with T'ang trachoma virus. At 24 hr after inoculation this culture was treated with tylosin tartrate 25 μg/cc for 2 additional days. In the original color transparency, the white sphere in the cytoplasm is stained red. Acridine orange stain and U.V. light × 640.

FIG. 2. FL cell infected with T'ang trachoma virus 3 days previously. No treatment. Viral material occupies a large cytoplasmic vacuole. In the original color transparency the colors induced in developing virus range from red to orange to mature green, pin-point elementary bodies. Acridine orange stain and U.V. light × 640.

could not be recovered from such cultures. Tylosin tartrate caused visible shrinkage of nucleoli, as did CTC, but was otherwise nontoxic at the doses used. 5. Addition of Tylosin $SO_4$ to infected cultures delayed maturation of virus for one day and thereafter the virus was indistinguishable from that in drug-free cultures.

*Discussion.* Except for streptomycin, antibiotics inactivated extracellular psittacosis virus(21) thus yielding results which were not representative of their clinical value. The same effect can be ascribed to trachoma agent. It is likely that drugs may be ineffective therapeutic agents because they fail to penetrate the wall of the infected cell, deteriorate rapidly, or are excluded from the biosynthetic mechanism of replication. Tests on psittacosis virus have shown a gradient of inhibitory effect among tylosin derivatives in which tartrate and gluconate were most effective, lactate was intermediate and sulfate was least effective(21).

The virus-inhibiting effects of CTC and of tylosin tartrate may be attributed to interference with the process by which the virus-oriented RNA matrix is mobilized or, at a preceding stage, with elaboration of viral DNA in the cell. This contrasts with the inhibitory effects of fluorinated pyrimidines after being applied to the "red ball" stage of psittacosis virus; incorporation of the drug into the synthetic sequence resulted in accumulations of visible masses of "fraudulent" virus-associated RNA or of incomplete viral DNA(28). Trachoma virus responds similarly to treatment with fluorinated pyrimidines(29). Thus, in addition to morphologic and serologic similarities among members of the psittacosis-LGV family of agents, the characteristic pattern of biosynthetic replication adds another bond to this relationship. This cytochemical sequence is unique and has been observed neither with rickettsial agents nor with vaccinia virus; therefore, it

TABLE I. Effects of Antibiotics on Replication of Trachoma Virus in McCoy Cells.

| Drug | Units/ml medium | Microscopic effect* | Virus recovery after treatment, particles/ml† |
|---|---|---|---|
| Streptomycin | 200 μg | Mature | $5.85 \times 10^6$ |
| Sulfanilamide | 200 " | " | $5.59 \times 10^6$ |
| Penicillin | 100 units | Partial inhibition | $1.04 \times 10^5$ |
| Chlortetracycline | 25 μg | Inhibition | Negative |
| Tylosin tartrate | 25 " | " | " |
| Tylosin $SO_4$ | 25 " | Mature | $9.1 \times 10^5$ |

* As observed with acridine orange stain and U.V. microscopy on day 3 after inoculation.
† As determined in tissue cultures(28).

may become another basis for identification of psittacosis and related agents.

It would be hazardous to transpose arbitrarily information from a laboratory procedure to clinical application; however, the experimental procedure has yielded results with psittacosis virus which reflected accurately the clinical value of drugs. The evidence presented here substantiates the ineffective role of streptomycin, penicillin, and sulfonamide in specific treatment of trachoma, confirms the therapeutic value of CTC, and endorses tylosin tartrate as a drug with sufficient merit to warrant clinical trial.

*Summary.* Six antibiotic drugs were tested for inhibitory effect on trachoma virus in human tissue cells. Chlortetracycline hydrochloride and tylosin tartrate interrupted the cytochemical sequence which reflected maturation of the virus. Penicillin and sulfanilamide only delayed maturation and streptomycin had no effect on the virus. A cytochemical procedure for detection of trachomastatic drugs is described.

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Received March 2, 1961. P.S.E.B.M., 1961, v107.

# Determination of Regional Blood Flow in Gastrointestinal Tract of Intact Animals. (26671)

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Lack of an *in vivo* method for measuring regional blood flow in the gastrointestinal tract has limited knowledge of gut physiology relative to this important parameter. As a consequence, the relationship of circulatory disturbances to local function and local pathology in the intact animal and man is relatively unexplored. It has been difficult to obtain precise information relating gut circulation to general circulatory dynamics and homeostasis. This preliminary report describes a radioisotopic technic devised to study regional circulation in the gut as applied to the descending colon.

*Method.* After a 48 hour fast, mongrel dogs were anesthetized with pentobarbital. The colon was washed with warm tap water until clean. A specially designed thin-wall glass (30 mg/cm$^2$) Geiger-Muller tube with a 5-inch anode length is encased in a closely machined aluminum sleeve 0.008 inch thick. A rounded tip for easy passage and conical shaped tailpiece to seal and ground the lead wires are cast in Epon.* The tube is passed a measured distance into the colon per rectum and its position is fixed by clamping the lead cable to the dog's tail.

Tube dimensions are 6 inches overall length and ¾ inch in diameter. A 4-foot rubber-covered wire cable extends from the tailpiece. One milliliter (containing 40 $\mu$c) of a rapidly cleared colloid incorporating a pure beta emitting radioisotope ($CrP^{32}O_4$) (1) is injected rapidly into a jugular vein. The blood dilution curve of the radioisotope is continuously monitored by the colon-probe connected to a counting rate meter (Nuclear-Chicago), set to the 0.5 second time constant. Counting rate is continuously registered on a recorder (Brown) with a frequency response of 0.3 second full scale. Cardiac output is simultaneously determined from the same bolus by means of a femoral artery dilution curve obtained by drawing blood through a polyethylene helix surrounding an externally shielded Geiger-Muller tube. The areas under the cardiac output curves are obtained by planometric integration. The counting rate of the tube is calibrated by aspiration of a known concentration of $CrP^{32}O_4$ through the helix surrounding the tube. Cardiac output (1/min) is then the quotient of injected radioactivity divided by the area under the extrapolated curve.

*Results.* Fig. 1 shows the results of occluding 3 small arteries supplying the region of the colon around the probe. The figures at the top show the isotope dilution curve in the femoral artery (cardiac output) on the left and the usual normal colon curve on the right before arterial occlusion (arrow at extreme lower right). The 2 arterial dilution curves (cardiac output) are quite similar, while the colon curve following arterial occlusion has changed considerably from the control curve. The initial peak has disappeared. The curve maximum appears seconds later than the initial peak and falls away slowly. This reduced and delayed curve probably represents limited blood flow from non-occluded collaterals, since lead foil shielding of the serosal surface of the moni-

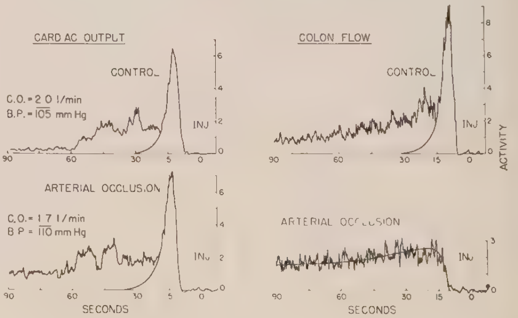

FIG. 1. Effect of local arterial occlusion on colon blood flow. Ordinate of cardiac output curve is concentration in arterial blood, cpm × $10^3$, in sample volume in helix. Ordinate of colon flow curve is activity, cpm × $10^3$, of blood in region of the colon probe.

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* Epon is Shell Chemical Co. epoxy resin (824) used with curing agent V.

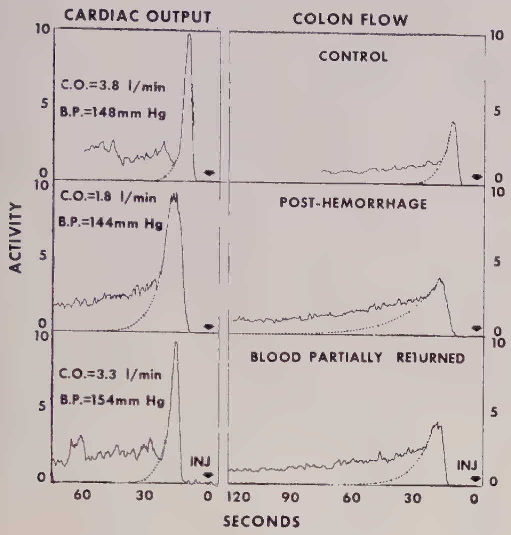

FIG. 2. Effect of hemorrhage on colon blood flow. Ordinate of cardiac output curve is concentration in arterial blood, cpm × $10^3$, in sample volume in helix. Ordinate of colon flow curve is activity, cpm × $10^3$, of blood in region of the colon probe.

tored colonic segment did not affect the colon dilution curve.

Fig. 2 illustrates the effect of hemorrhage (which reduces gut blood flow) on the shape of the colon curve. The upper curves are normal control curves before hemorrhage. The curves in the middle were obtained after removing 250 ml of blood (15 ml/kg). The ascending limb of the colon curve is slowed, peak is delayed and slightly decreased in height; descending limb shows delayed washout of the isotope. The cardiac output has fallen from 3.8 l/min to 1.8 l/min. The lower curves were obtained after replacing one-half (125 ml) of the blood. They demonstrate a partial return toward the control curves. After all the blood was returned (not shown) the curves do not differ from the top control curves.

Fig. 3 shows an experiment to study the effect of local venous occlusion. After moderate hemorrhage (to reduce colon blood flow), umbilical tapes were placed around 3 veins draining the region of the colon around the probe. The uppermost colon flow curve was obtained after hemorrhage (8 ml/kg) and placement of umbilical tapes around the veins without occlusion. The post hemorrhage control curve has slower than normal ascending and descending limbs and a

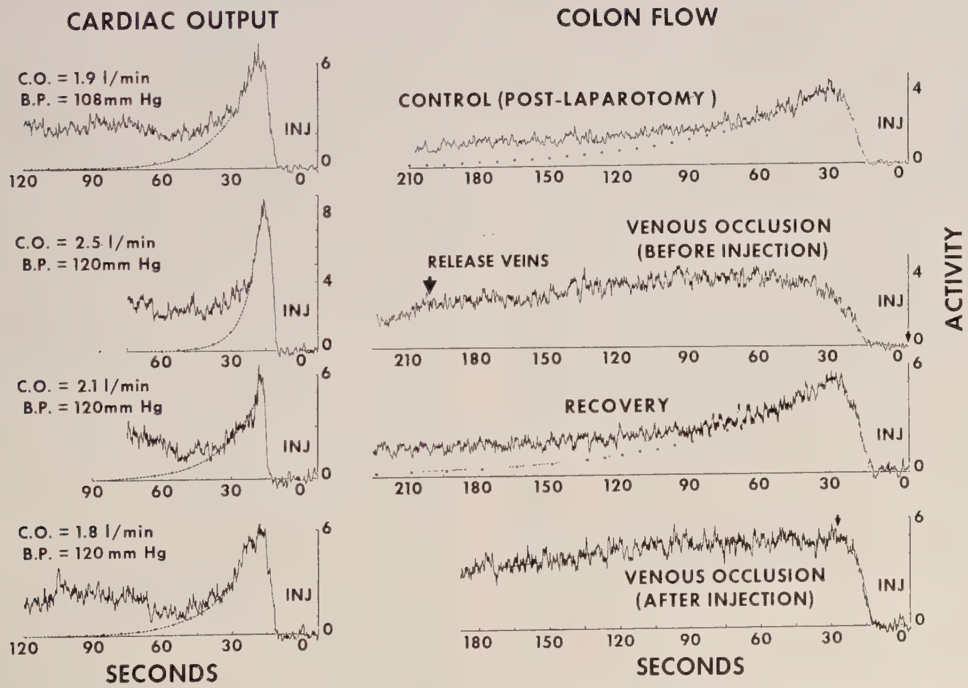

FIG. 3. Effect of local venous occlusion on colon blood flow. Ordinate of cardiac output curve is concentration in arterial blood, cpm × $10^3$, in sample volume in helix. Ordinate of colon flow curve is activity, cpm × $10^3$, of blood in region of the colon probe.

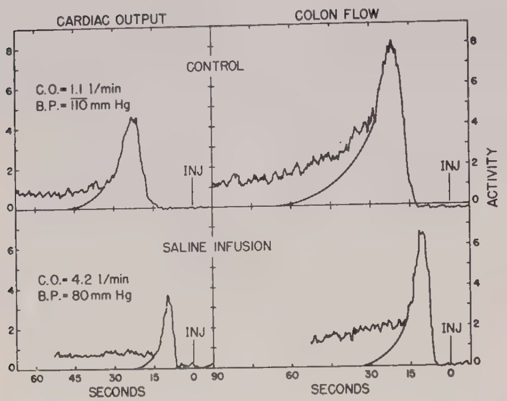

FIG. 4. Effect of saline infusion on colon blood flow. Ordinate of cardiac output curve is concentration in arterial blood, cpm × $10^3$, in sample volume in helix. Ordinate of colon flow curve is activity, cpm × $10^3$, of blood in region of colon probe.

rounded peak resembling the middle curve in Fig. 2. The second colon curve was obtained when the injection of the isotope was made 5 minutes after the veins were occluded. Circulation time is unchanged, the ascending limb of the curve is slowed still further, the peak is delayed, plateau prolonged, and washout delayed until the veins are released after which there is a sharp increase in the washout slope (2nd curve, release veins). Cardiac output was slightly increased. The third colon curve was obtained 25 minutes after the veins had been released (recovery) and shows a return to control curve pattern. The bottom colon curve was obtained by injecting the bolus of isotope and occluding the veins (arrow) after the curve had reached its peak. From this curve it is clear that blood containing the isotope entered the colon region without difficulty but sudden venous occlusion traps the blood and retards its exit.

Fig. 4 shows an experiment in which 1 liter of saline was infused after the control curves were obtained. After saline infusion the cardiac output increased from 1.1 liter per minute to 4.2 liter per minute. Circulation time to the colon is shorter and the changes in the colon curve are similar to those seen in the arterial dilution curve. Both ascending and descending limbs of the colon curve are steeper than those in control curve and probably represent increased blood flow to the colon.

*Discussion.* The range of the $P^{32}$ beta particle in tissue is obtained by centering the colon probe in a series of calibrating cylinders of increasing radius and filling each cylinder with an aliquot of $P^{32}$ solution. It is found that 73% of the maximal counting rate is the result of the radioactivity present within 1 mm of the outer surface of the probe, 94% of maximal counting rate (infinite volume) lies within 4 mm of the probe surface. Because the range of the beta particle in tissue is very short ($P^{32}$ half thickness 0.8 mm) the volume of blood monitored by the colon probe is virtually limited to the segment of colon surrounding its anode length. Incorporation of the beta particle into a colloid retains the isotope intravascularly until removed by the reticuloendothelial system.

The cardiac output as represented by the arterial dilution curve is the input function. The simultaneously determined colon flow curve is the resultant of the input function of isotope concentration (cardiac output) modified by local vascular factors in the region of the colon monitored by the probe. The colon curves represent several aspects of regional circulation: (a) circulation and transit times, (b) fraction of cardiac output perfusing the monitored region, (c) rate of local blood turnover.

As yet blood flow has not been quantitated and interpretation of the regional colon flow curves is based upon experiments which alter regional flow in a predictable way such as local arterial or venous occlusion and hemorrhage which decrease flow, and saline infusion which increases regional colon flow above normal. In a number of experiments simultaneous timed collections of the regional venous outflow verified directly the changes in regional blood flow.

While at present the procedure is qualitative, the curve shapes are nevertheless a sensitive index of directional change in blood flow in the region under study, where the animal serves as its own control. This procedure is being used to study the circulatory parameters of physiological, pharmacological and pathological interest in the colon.

*Summary.* A technic is described for estimating regional blood flow in the gastrointestinal tract. This method utilizes the short range of the beta particle in tissue to limit the blood volume monitored by a special Geiger-Muller tube placed in the intestinal lumen.

Received March 10, 1961. P.S.E.B.M., 1961, v107.

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## Corticotropin Releasing Activity of Synthetic Lysine Vasopressin.* (26672)

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Our attempts to isolate a corticotropin releasing factor (CRF) from extracts of porcine posterior pituitary have been complicated by the facts that CRF is apparently quite difficult to separate from vasopressin, and that vasopressin itself has been shown to possess corticotropin (ACTH) releasing activity. When doing CRF bio-assays on fractions containing vasopressin it is necessary to take into account any "CRF" activity due to the vasopressin present. In this paper we report our values for ACTH-releasing activity of synthetic lysine vasopressin, using the same *in vivo* CRF bio-assay(1) used in our isolation work.

*Materials and methods.* Synthetic lysine vasopressin was kindly provided by Dr. V. du Vigneaud, Dept. of Biochemistry, Cornell Univ. Medical College. The sample used in these experiments (JM V 68/16) was reported to contain 260 International Units (U)/mg. Two assays of this sample *vs* USP Posterior Lobe Reference Standard in dibenamine treated rat by method of Dekanski(2) gave us 258 (95% fiducial limits: 228-291) and 267 (95% fiducial limits: 256-279) U/mg, respectively; the mean of these 2 assays, 262 U/mg, was taken as the pressor activity of the sample. The entire solid sample as received was dissolved in deionized water for pressor assay, the solution then divided into aliquots and each of these lyophilized and stored in deep freeze; assays for CRF could then be carried out on a solid sample freshly diluted in physiological saline.

For CRF assays, 150-170 g male rats from Holtzman Co., Madison, Wisc. were used. Only modification of the *in vivo* assay procedure of Guillemin(1) was that morphine was injected 35 min after nembutal injection instead of 25 min. Uninjected rats and rats injected with 0.3 ml physiological saline instead of vasopressin were introducd randomly throughout each assay as controls. Release of ACTH was determined by measuring concentration of free corticosteroids in 2 ml of plasma by the method of Silber as modified by Guillemin(3). In rats, corticosterone (cpd B) is the principal steroid determined.

In several experiments, male Holtzman rats of the same weight range were hypophysectomized and 24 hrs later injected with the vasopressin sample; these rats were not treated with nembutal-morphine but were anesthetized with ether before injection of sample. After 15 min, blood was drawn and plasma corticosteroids were determined as in the standard CRF assay.

*Results* are shown in Table I. Controls in each case include all values from both uninjected and saline injected rats, since statistical analysis of the two types of control values showed no significant difference between them. Data for the 3 dose levels of vasopressin giving significant increases of

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* This investigation has been supported in part by a grant-in-aid from Eli Lilly & Co. and by research grant from Nat. Inst. of Neurol. Dis. and Blindness, U.S.P.H.S. Journal Paper No. J-4022 of Iowa Agri. and Home Economics Exp. Station, Ames. Project No. 1304.

TABLE I. Effect of Synthetic Lysine Vasopressin on Plasma Corticosterone of Rats.

| | Controls | | Inj. rats | | | |
|---|---|---|---|---|---|---|
| Pressor amt of LVP in mU | No. of rats | Plasma-cpd B, μg/100 ml | No. of rats | Plasma-cpd B, μg/100 ml | p value† | % increase over controls |
| 8.4 | 7 | 8.0 ± .8* | 10 | 8.5 ± 1.1 | $.8 > p > .7$ | — |
| 16.8 | 18 | 8.2 ± .7 | 19 | 11.4 ± .7 | $.01 > p > .001$ | 39 |
| 33.5 | 10 | 8.1 ± .8 | 9 | 14.7 ± 1.4 | $< .001$ | 81 |
| 67 | 7 | 8.1 ± 1.4 | 10 | 18.2 ± 1.4 | $< .001$ | 125 |
| | | | Hypophysectomized rats | | | |
| 67 | 11 | 2.4 ± .1 | 9 | 2.5 ± .1 | $.8 > p > .7$ | — |

* Mean ± stand. error.

† p calculated from t on values for plasma-cpd B in controls and in sample-injected animals.

plasma corticosteroids over controls were also submitted to analysis of variance, giving a value of 11.4 for F, significant at the 0.1% level. Fig. 1 shows the regression line for percent increase of plasma corticosteroids *vs* log of dose of vasopressin and the 3 experimental points with standard errors of the means. The regression equation is: $y = -134 + 141.5\ (x)$.

In the hypophysectomized animals, control values were lower than in nembutal-morphine treated animals, but injection of as much as 67 mU of synthetic lysine vasopressin caused no increase whatever over controls.

*Discussion.* McCann and Fruit(4) have also investigated the ACTH releasing activity of synthetic lysine vasopressin, using rats with acute hypothalamic lesions as experimental animals and depletion of adrenal ascorbic acid as index of ACTH release. Synthetic vasopressin was found to give the same log dose *vs* response curve given by commercial Pitressin in the range of 62 to 1,000 mU of pressor activity. Minimal effective dose was of the order of 100 mU in McCann's system.

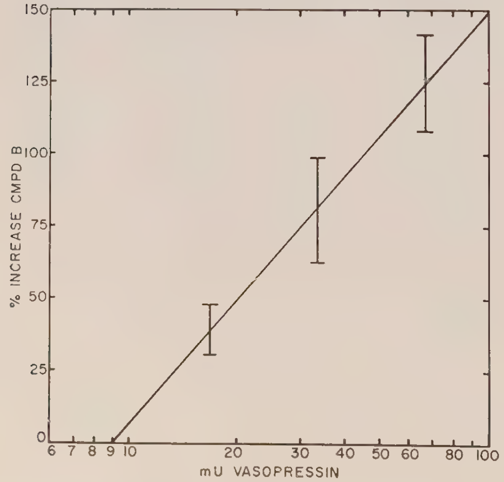

FIG. 1. Increase of plasma corticosterone (± S.E. of mean) in nembutal-morphine blocked rats *vs* mU synthetic lysine vasopressin injected; (logarithmic abscissa).

Guillemin *et al.*(1) more recently have examined ACTH releasing activity of lysine vasopressin in the nembutal-morphine blocked rat, using a sample of vasopressin isolated from porcine pituitary preparations. Their sample had been purified by the method of Ward and Guillemin(5) and further purified by paper chromatography in *m*-cresol/water to a pressor activity of 287 U/mg. They used much lower doses than McCann and Fruit, down to 6 mU, but concluded that there is a threshold for ACTH release by vasopressin at approximately 60-80 mU of pressor activity. That is, above about 80 mU a large discharge of ACTH was observed, increasing linearly with log dose of vasopressin, but below 80 mU, no statistically significant release of ACTH could be detected. Guillemin used increase of plasma corticosteroids determined fluorometrically as his index of ACTH release and found this to be a more sensitive index than adrenal ascorbic acid depletion when both determinations were done on the same animal.

In contrast to Guillemin's report, our results, using synthetic lysine vasopressin, show an excellent linear log dose *vs* response relationship all the way down to about 8-10 mU of pressor activity, using essentially Guillemin's experimental conditions. From our results it appears necessary for us to dilute unknown samples down to a pressor dose of about 10 mU before assaying for CRF in the nembutal-morphine treated rat, instead of the 80-100 mU threshold indicated by Guillemin(6); in his laboratory, samples are actually diluted down to 30 mU for CRF assay(7). Alternatively, it may be legitimate to subtract the ACTH releasing activity corresponding to the amount of vasopressin injected (as determined by pressor assay). When assaying for CRF near a pressor peak in fractionations by chromatography, electrophoresis, or counter current distribution, the value taken for the ACTH releasing activity intrinsic in vasopressin assumes critical importance.

It is always possible to confuse ACTH releasing activity with activity of ACTH itself unless the latter is specifically ruled out. Different investigators, using a variety of vasopressin preparations, experimental animals, and indices of "ACTH-like" activity, have come to opposite conclusions about direct action of vasopressin on the adrenal cortex. Thus, Sobel *et al.*(8) found that urinary corticosteroids increased after injection of commercial Pitressin in normal guinea pigs, but not in hypophysectomized guinea pigs. Several figures in a paper by Schally and Guillemin(9) indicate that chromatographically purified arginine vasopressin and lysine vasopressin did not increase plasma corticosteroids on injection in hypophysectomized rats. On the other hand, Royce and Sayers(10) found that large doses (2.5-5.0 U) of purified arginine vasopressin or of commercial Pitressin depleted adrenal ascorbic acid in hypophysectomized rats, and Hume and Nelson reported that *i.v.* injection of vasopressin increased 17-hydroxycorticoids in adrenal venous blood of hypophysectomized dogs(11). Hilton *et al.*(12) recently described a direct ACTH-like effect of synthetic vasopressins, measured by increase of adrenal venous hydrocortisone during direct arterial perfusion of adrenals of hypophysectomized dogs. Our negative results with injection of synthetic vasopressin in hypophysectomized rats indicate that the effect we have observed is not "ACTH-like" but rather a true "CRF-like" effect.

*Summary.* Synthetic lysine vasopressin was assayed for ACTH releasing activity in nembutal-morphine treated rats using fluorometric determination of plasma corticosteroids as index of ACTH release. A linear log dose *vs* response relationship was established between pressor doses of 8 to 67 mU. This activity was shown to be "CRF-like" rather than "ACTH-like" by its absence in hypophysectomized rats injected with 67 mU of pressor activity.

The authors wish to express appreciation to Dr. Stephen Mandel, Dept. of Statistics, I.S.U., for many helpful discussions during this work.

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Received March 22, 1961. P.S.E.B.M., 1961, v107.

# Immunization of Mice with Inactivated Herpes Simplex Virus.[*] (26673)

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While several reports indicate that inactivated herpes simplex virus is effective for immunization of experimental animals(1-4), recent articles cast doubt as to its efficacy (5-7). The immunologically related herpes B virus, after inactivation with formalin, is of low potency as an immunizing agent for its natural host, the monkey, but produces effective immunity in rabbits(8). As herpes simplex virus infection is a prototype of mammalian virus latency, it is especially desirable to understand its immunogenic characteristics. The evidence presented here indicates that immunization of mice with noninfective virus may be accomplished with the production of neutralizing antibody in this host.

*Materials and methods.* The strain of herpes simplex virus used was isolated on chick embryo fibroblast culture in this laboratory in Jan., 1957, from a herpes simplex labial lesion and has undergone 12 serial passages on chick embryo fibroblast or rabbit kidney cell monolayer cultures. It is neutralized by rabbit herpes simplex antiserum.

Male CFW albino mice of 18-20 g weight obtained from Carworth Farms, New City, Rockland Co., N. Y. were used throughout these experiments.

Neutralizing antibody titers are expressed as that dilution of serum neutralizing 100 $TCD_{50}$ or 10 $TCD_{50}$ of herpes simplex virus on human liver cell cultures. Titers were determined for individual animals and no dilution below 1:8 was tested because of the small amount of serum available.

Procedures and materials varied in some respects in each of 3 mouse immunization experiments (Table I). The virus was grown on rabbit kidney cell monolayers with a maintenance medium of 2% calf serum in 199[†] and containing 100 units of penicillin and 100 μg of streptomycin per ml. This tissue culture fluid was found to have a titer of $10^{5.5}$ $TCD_{50}$/ml on human liver cell cultures in Exp. I. The titers were $10^{7.0}$ and $10^{7.5}$ $TCD_{50}$ respectively on rabbit kidney cell cultures in Exp. II and III. Titration end points of herpes simplex virus are 1.5 and 2.0 logs higher with rabbit kidney than in liver cell culture. Hence, these 2 virus suspensions were of approximately equal initial infectivity titer.

Virus was inactivated in 10 ml lots in a 9.5 cm Petri dish, using a Westinghouse Sterilamp G8T5 (u.v. germicidal lamp), at 4 inches distance for 13 minutes. Subsequent inoculation of this preparation undiluted or in $10^{-1}$ dilutions on rabbit kidney monolayers gave no cytopathic effect. Cultures in thioglycollate broth and in blood agar showed no bacterial growth. Uninoculated maintenance medium was removed from rabbit kidney cell monolayers and irradiated for use as a control in Exp. III.

*Results.* (Table I) in Exp. I, 2 groups of 5 mice each received 0.2 ml of inactivated virus into the tail vein followed in 11 days by 0.5 ml of inactivated virus intraperitoneally. Intracerebral challenge with 333 $LD_{50}$ or 33 $LD_{50}$ or herpes simplex virus caused no deaths in either immunized group. None of the control mice which received 333 $LD^{50}$ and only one control mouse injected with 33 $LD_{50}$ survived.

In Exp. II, 2 groups of 15 mice immunized as above, were injected intracerebrally with herpes simplex virus in a calculated dosage of 1700 $LD_{50}$ or 170 $LD_{50}$. Only one death occurred in each group of immunized mice while 14 deaths occurred in 15 control mice which received 1700 $LD_{50}$ and 8 of 15 unimmunized mice died which received 170 $LD_{50}$. It was surprising that only 8 of the 16 control mice died from the calculated 170 $LD_{50}$ dose. However, this $LD_{50}$ had been deter-

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[*] This investigation was supported in part by grants from Nat. Inst. Health, Division of Allergy and Infect. Dis., and from Am. Cancer Soc.

[†] Microbiological Associates, Inc., Bethesda, Md.

TABLE I. Immunization of Mice with Inactivated Herpes Simplex Virus.

| Exp. | Antigen | Intracerebral challenge ($LD_{50}$) | S/T* | % |
|---|---|---|---|---|
| I | Inact. HSV† | $3.3 \times 10^2$ | 5/5 | 100 |
| | None | $3.3 \times 10^2$ | 0/5 | 0 |
| | Inact. HSV | $3.3 \times 10^1$ | 5/5 | 100 |
| | None | $3.3 \times 10^1$ | 1/5 | 20 |
| II | Inact. HSV | $1.7 \times 10^3$‡ | 14/15 | 93 |
| | None | $1.7 \times 10^3$‡ | 1/15 | 7 |
| | Inact. HSV | $1.7 \times 10^2$‡ | 15/16 | 93 |
| | None | $1.7 \times 10^2$‡ | 8/16 | 50 |
| III | Inact. HSV | $10^3$ | 10/14 | 71 |
| | Cell culture fluid | $10^3$ | 0/12 | 0 |

* S/T = Survivors/Total experimental animals.
† Herpes simplex virus.
‡ $LD_{50}$ previously determined in younger mice (see text).

mined 3 weeks earlier on mice of the same shipment, and it has been shown that susceptibility of mice to herpes simplex virus infection decreases significantly with age(9).

In Exp. III, a group of 15 mice immunized by the schedule previously described were challenged intracerebrally 28 days after the second injection with 1000 $LD_{50}$ of herpes simplex virus. A group of 15 control mice were injected with irradiated cell culture fluid and similarly challenged. One mouse of the immunized group and 3 control mice died of undetermined cause prior to intracerebral challenge. Ten of 14 immunized mice survived while all 12 control mice died within 7 days.

A second group of 15 immunized mice and 15 control mice was bled for antibody determination 28 days after completion of immunization. Sufficient serum for antibody studies was obtained from 14 of the immunized mice and 9 of the control mice. No antibody could be demonstrated against 100 $TCD_{50}$ of herpes simplex virus, although there was delayed viral cytopathic effect in the presence of serum from immunized mice as compared to serum from control mice. Neutralizing antibody was demonstrated against 10 $TCD_{50}$ of herpes simplex virus in all but one of the 14 immunized mice tested. None of the 9 control sera had neutralizing activity against herpes simplex virus.

*Discussion.* The successful attempts at herpes immunization reported earlier were done with virus grown in intact animal hosts and inactivated with formalin or phenol. In our experiments the use of ultraviolet inactivated virus from relatively simple cell culture fluids obviated the introduction of animal tissue and formalin into the immunizing materials.

In only 2 instances was neutralizing antibody demonstrated by previous investigators following immunization with inactivated virus, both times in the guinea pig(2,10). We were able to demonstrate antibody in mouse serum as might be expected, but this has not been reported by others.

Comparison of Exp. I and II with Exp. III (Table I) suggests that the immunity conferred by inactive virus may fall rapidly. Ninety to one hundred per cent survival was seen in animals challenged one week after completion of immunization, while only 71% survived when challenged 28 days after completion of immunization. In earlier studies the challenge interval has been only 10 days to 2 weeks.

It is suggested that cell culture materials may confer a greater immunity than is seen with antigen from animal sources. Animals immunized with inactivated virus from animal brain or skin lesions withstood 10 to 100 MLD while 71% of those immunized with inactivated cell culture virus were able to withstand 1000 $LD_{50}$ in the present study. This may be related to the higher pre-inactivation titers of the cell culture virus suspensions. While our studies were in progress a report of similar findings appeared in which mice immunized with inactivated herpes simplex virus from rabbit kidney cell culture fluid withstood a challenge of 10,000 $LD_{50}$ (10). There are several possible explanations of the failure of inactivated herpes simplex virus produced in eggs to induce immunity. Jawetz, Allende and Coleman(6, 7) employed material of lower pre-inactivation virus titer than is obtainable in cell culture materials. Hence the amount of antigen injected may have been less than that in cell culture materials. A second possibility is that the inactivation procedures used may have destroyed antigenicity as well as infectivity. It is also possible that along with the well recognized alteration in virulence of herpes simplex virus on egg passage, there is some accompanying change in antigenic potency. In fact, a recent report indicates that herpes virus grown in eggs may be less active antigenically than virus from cell culture or mouse brain even if pre-inactivation titers are comparable(10).

It is clear from our experiments and those of Chu and Warren(10) that injection of inactivated herpes simplex virus in mice can protect against a large intracerebral challenge. Production of demonstrable neutralizing antibody also results from these injections.

*Summary.* Ultraviolet inactivated herpes simplex virus prepared in rabbit kidney cell cultures was injected intravenously and intraperitoneally into mice. The majority of mice so immunized survived a 1000 $LD_{50}$ intracerebral herpes simplex virus challenge and were shown to possess neutralizing antibody against the virus. These findings are contrary to the findings of studies in which inactivated allantoic fluid virus was used as antigen. However, they are in agreement with early experiments which employed inactivated herpes simplex virus prepared in mouse or rabbit brain and a recent report in which antigen was also prepared in rabbit kidney cell culture.

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Received March 23, 1961. P.S.E.B.M., 1961, v107.

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# Effect of Hyperthyroidism on Body Weight Gain and Feed Consumption in Male and Female Rats.[*] (26674)

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Normal thyroxine secretion rates (TSR) of many female rats of Sprague-Dawley-Rolfsmeyer (S-D-R) strain have been determined in this laboratory(1,2). TSR varied from 0.25 μg to 2.0 μg/100 g B W, with mean of about 1.0 μg. A significant increase in TSR was observed in lactating rats(3). Lactating rats showed a range up to 3.0 μg. Use of 3.0 μg/100 g B W has been shown to stimulate significant increases in mammary gland growth(4,5) and lactation(6). Unpublished data on TSR of normal male rats of this strain show a range of 0.5 μg to 1.5 μg/100 g B W with a mean of about 1.0 μg/100 g B W.

The object of the present experiment was to relate mean and maximum TSR of this strain of rats with optimal level of hyperthyroidism for weight gain. It was considered that injection of graded levels of thyroxine above mean and maximum TSR would be reflected in changing weight gain in such a manner as to indicate optimal hyperthyroidism and transition to a state of thyroxine induced stress.

*Material and methods.* Male and female

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[*] Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2288. Approved by Director.

[†] This investigation was supported in part by a grant from U. S. Atomic Energy Commission.

TABLE I. Influence of Increasing Levels of Thyroxine on Body Weight Gains of Male and Female Rats.

| Thyroxine level (μg/100 g BW) | | Days of treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | Start | 20 | 40 | 80 | 120 | 160 | 200 |
| | Avg body wt (g) | | | | | | |
| Control ♂ | (7)*45 | (7) 160.7 | (7) 274.4 | (6) 387.8 | (5) 448.8 | (5) 472.4 | (5) 506.6 |
| ♀ | (11) 44 | (11) 137.8 | (10) 192.2 | (10) 248.2 | (10) 265.2 | (10) 271.3 | (10) 279.9 |
| 3.0 μg ♂ | (7) 44 | (7) 160.7 | (7) 257.2 | (4) 341.0 | (4) 378.5 | (4) 396.5 | (4) 398.0 |
| ♀ | (11) 38 | (10) 112.7 | (9) 181.1 | (9) 228.4 | (9) 242.8 | (9) 260.2 | (9) 266.2 |
| 6.0 μg ♂ | (9) 37 | (9) 154.2 | (9) 248.7 | (9) 314.0 | (7) 328.8 | (8) 317.6 | (5) 308.0 |
| ♀ | (9) 38 | (8) 131.0 | (8) 187.5 | (7) 222.0 | (7) 249.0 | (7) 263.0 | (7) 271.8 |
| 9.0 μg ♂ | (9) 45 | (7) 153.8 | (7) 241.5 | (6) 301.5 | (6) 321.5 | (6) 345.5 | (5) 362.0 |
| ♀ | (9) 44 | (9) 124.4 | (9) 185.5 | (9) 224.1 | (9) 239.0 | (8) 245.8 | (4) 260.7 |
| 12.0 μg ♂ | (9) 51 | (9) 152.5 | (9) 233.1 | (7) 297.7 | (6) 319.0 | (3) 342.2 | (3) 331.5 |
| ♀ | (9) 47 | (9) 133.1 | (9) 191.5 | (9) 227.4 | (9) 247.7 | (9) 253.7 | (9) 263.3 |

* No. of animals in parentheses.

rats of Sprague-Dawley-Rolfsmeyer (S-D-R) strain, age 26 days, were injected daily with 3.0 μg, 6.0 μg, 9.0 μg, and 12.0 μg L, thyroxine/100 g body weight for 200 days. Control group was injected with an alkaline saline solution. Body weight was recorded at regular intervals. Commercial lab feed (Purina) was supplied *ad libitum*. Animal room was maintained at uniform temperature of 78 ± 1°F.

When animals were mature, accurate feed consumption records were kept for a period of 12 days. Daily feed consumption was expressed as g/100 g B W.

*Results.* In relation to normal thyroxine secretion rate in females, thyroxine ($T_4$) at 3 μg/100 g B W is about 3 times mean secretion of this strain and about ⅓ above maximum secretion rate. In comparison to lactating rats, 3 μg $T_4$ is equal to maximal level observed.

Female animals injected with 3 μg $T_4$ weighed 82% of controls at 20 days, and 94, 92, 92, 96 and 95% of the controls at successive 40-day intervals up to 200 days (Table I). In successive groups of females, body weights were reduced no more than 10% below the controls in any age group. As level of $T_4$ was increased no trend toward greater weight reduction was observed.

In males, injection of 3 μg $T_4$ began to influence body weight gains at 40 days, and continued to 200 days with a reduction to 79% in comparison to controls at that time. Similarly, at 6 μg $T_4$ weight reduction was increased to 61% of controls, at 9 μg to 71%, and at 12 μg to 65% of the controls. Thus, male rats were more severely rstricted in body weights by progressive degrees of hyperthyroidism than were females. Further, females survived the treatment much better than did males.

Mean food consumption per 100 g B W of control animals was 4.9 g for males and 5.4 g for females. As $T_4$ level increased, food consumption progressively increased by 37% in females and over 70% in males.

*Discussion.* In past experiments, physiological effects of thyroxine administration have been reported in many types of experimental animals. In many cases, amount of thyroxine injected was excessive in relation to recently observed normal TSR and question could be raised whether results indicated physiological effect of thyroxine or a stress-induced effect. With development of a quantitative method of estimating TSR and availability of data indicating mean and

TABLE II. Influence of Increasing Levels of Thyroxine upon Mean Daily Feed Intake.

| Thyroxine level (μg/100 g BW) | Mean daily feed consumption (per 100 g body wt) | | | |
|---|---|---|---|---|
| | ♂ | | ♀ | |
| | g | %* | g | %* |
| Control | 4.9 | | 5.4 | |
| 3.0 | 5.6 | 14.2 | 6.0 | 11.1 |
| 6.0 | 7.2 | 46.9 | 6.5 | 20.3 |
| 9.0 | 8.5 | 73.4 | 7.2 | 33.3 |
| 12.0 | 8.4 | 71.4 | 7.4 | 37.0 |

* % increase over controls.

range of normal TSR of both sexes in S-D-R strain of rats, it seemed desirable to determine optimal range of hyperthyroidism (in relation to mean and maximum TSR) and levels where stressful effects begin to appear.

In previous studies it was shown that 3 μg $T_4$/100 g B W significantly stimulated growth of mammary gland(4) and intensity of milk secretion(6) in this strain. This level was considered optimal for these physiological processes in mature animals. In present study, it is shown that this level depressed body weight gains of females temporarily, but weight was largely regained at later age intervals. More surprising, however, was the observation that 2, 3, or 4 times this level of $T_4$ had no greater effect upon depression of body weight. This indicates that in females up to 12 times mean TSR level is well tolerated by growing rats. It indicates, also, that mild hyperthyroidism has no stimulating effect upon rate of weight gain.

In Long-Evans strain of rats, Evans *et al.* (7) noted a differential in biological effectiveness of thyroxine after thyroidectomy. Nearly normal growth was supported in females at 0.25 μg and at 0.50 μg growth was within normal limits.

In male rats, effect of $T_4$ was minimal during first 20 days of administration; thereafter its depressing effect upon body weight was progressive until end of experiment. Level of $T_4$ of 6 to 12 μg/100 g B W was quite similar in its depressing effect upon body weight. These levels depressed body weight far more in males than in females.

Present results are in general agreement with Koger and Turner(8) who observed depressions in body weights of male and female rats following oral administration of increasing levels of thyroprotein to S-D-R strain. Nose-anus length of males was slightly greater than controls in spite of lower body weights.

Causes of differences in weight gain of 2 sexes is obscure. It has been suggested that TSR might play a role. Data indicate that mean TSR of 2 sexes of normal animals is similar. Further, levels of $T_4$ above normal are without benefit on weight gain of females. In males, higher levels of $T_4$ tend to depress body weight more severely than in females. Clearly, in rats, $T_4$ plays no role in sex difference in rate of gain nor in mature body weight.

It has long been recognized that hyperthyroidism stimulates appetite and increases feed intake. Present data confirm that observation. Not recognized, heretofore, is sex difference. Females were capable of maintaining more nearly normal weight gain on higher levels of $T_4$ yet consumed less additional feed than did males.

It is possible that sex differences exist in regard to metabolism of $T_4$. In cows it has been shown that t½ of $T_4$ is reduced about 54% when $T_4$ was injected 50% above TSR (9). Observed differences in sexes in present study might be explained, in part, if t½ of $T_4$ in hyperthyroidism is shown to differ in sexes of rats.

No evidence of a $T_4$ induced stress was observed in females of this strain when up to 12 times mean TSR was administered. In males, levels of 6 times or more severely restricted body weights with advancing age.

*Summary.* Male and female rats of Sprague-Dawley-Rolfsmeyer strain, age 26 days, were injected daily with 3.0, 6.0, 9.0, and 12.0 μg L, thyroxine/100 g B W for 200 days in comparison with normal groups. Lowest level was 3 × mean TSR and equal to highest rate observed in lactating rats. In females, weight gain was reduced by less than 10% in comparison with controls with increasing age, and no trend toward greater weight restriction was observed on higher levels of $T_4$. In males, increased weight restriction appeared at 40 days and beyond, and was more marked than in females. Levels of 6 μg and above permitted gain equal to 60 to 70% of that of controls. Food consumption increased progressively on higher $T_4$ levels but females showed less increase than did males.

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Received April 14, 1961. P.S.E.B.M., 1961, v107.

# Determination of Alpha-glycerophosphate Oxidation with Tetrazolium.* (26675)

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A variety of technics are presently employed for assay of alpha-glycerophosphate dehydrogenases, but none are entirely satisfactory in terms of precision or reproducibility. Growing emphasis on the significance of glycerophosphate oxidation in muscle metabolism(1), endocrine dysfunction(2), and the biochemical characteristics of tumors(3) indicates the need for a simple and reliable technic for assaying the enzyme systems involved in oxidation of glycerophosphate. INT (2-p-iodo-3-p-nitro-5-phenyl tetrazolium chloride), a monotetrazolium salt, has been used successfully for measuring a number of enzyme systems(4,5). The present communication describes and evaluates a simplified technic utilizing INT for determination of the activities of the soluble (DPN-dependent) and insoluble (cytochrome-dependent) alpha-glycerophosphate oxidase complex (AGPO) and the corresponding specific alpha-glycerophosphate dehydrogenases (AGPD) in homogenates of animal tissues.

*Method.* Excised tissues of V stock house mice (Bar Harbor) were placed in iced Ringer solution, and homogenates of appropriate strengths made up with deionized distilled water. Homogenization was carried out with motor-driven teflon pestles in glass tubes. Total AGPO was assayed in 10 ml test tubes containing 0.2 ml of 0.5M AGP (alpha-glycerophosphate, Sigma), 0.3 ml of 0.4% INT (Dajac), 0.2 ml of 1% DPN (Sigma), and 0.2 ml of 0.5% or 1.0% homogenate. M/15 phosphate buffer (pH 8.0) was added to bring the total volume to 2.0 ml. In assays for insoluble AGPO, DPN was omitted. Corresponding assays for total and insoluble AGPD were carried out by adding 0.2 ml of $10^{-4}$M phenazine methosulfate (Sigma) and 0.2 ml of $10^{-3}$M NaCN to reaction mixtures before bringing to a final volume of 2.0 ml. This step establishes direct electron transfer between the dehydrogenase and INT. Appropriate blanks containing no substrate were run in all experiments. After uniform agitation (by holding against a rotating cuboidal rubber stopper for 10 seconds) the tubes were placed in a water bath at 37°C for 15 minutes. The reaction was stopped with 0.2 ml of 30% TCA. Prolonged contact with the acid should be avoided, as a 5% loss of formazan was found after 24 hours exposure to the TCA. The iodoformazan (IF) produced during incubation was extracted with 5 ml ethyl acetate, shaken vigorously 50 times, centrifuged, and the decanted ethyl acetate extract read spectrophotometrically (Coleman Jr.) at 490 m$\mu$ against a calibration curve of chemically reduced (hydrosulfite) IF in ethyl acetate. Total nitrogen values in equivalent aliquots of homogenate were determined by the micro-Kjeldahl method and enzyme activities expressed as $\mu$g IF per $\mu$g tissue N. Values for DPN-dependent enzyme activity were obtained by subtracting the values for the in-

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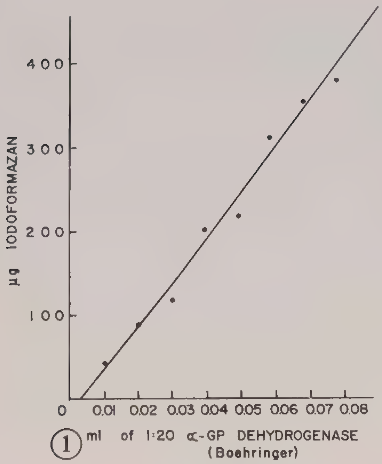
* This investigation has been aided by a grant from Nat. Inst. Health, U.S.P.H.S.



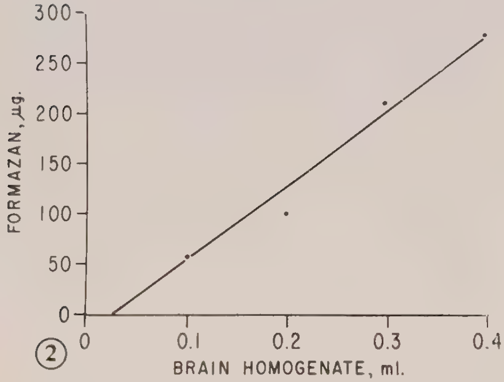


FIG. 1. Production of iodoformazan by varying amounts of crystalline alpha-glycerophosphate dehydrogenase.

FIG. 2. Increase in formazan production with increasing volumes of 5% rat brain homogenate.

soluble component from total activity levels.

Varying amounts of a commercial (Boehringer) crystalline preparation of AGPD (soluble) were assayed and linearity established between IF produced and amount of enzyme used (Fig. 1). Linearity of a similar order was also obtained with a series of varying levels of tissue homogenates (Fig. 2). The course of the reaction was linear up to 30 minutes, except at very low levels of enzyme activity. In several runs iodoacetate was added to a final concentration of 0.001M in order to block any phosphoglyceraldehyde dehydrogenase activity which might conceivably be contributing to the enzyme activities measured. Since no appreciable effect was observed with this inhibitor it can be assumed that the assay is specific for the enzymes involved in glycerophosphate oxidation only.

*Results.* In experiments with homogenates from male V stock house mice the order of decreasing activity of total (soluble and insoluble) AGPO was liver>kidney>brain>muscle (Table I). For total AGPD the order of activity was kidney>liver>muscle>brain. The insoluble AGPO and AGPD levels were negligible or low in all tissues except brain. In tissues other than brain the total AGPO and AGPD activities reflect the soluble enzyme components almost completely. In brain the insoluble enzyme constituted about 70% of total AGPO and AGPD activity. In selected instances determinations of tissue activity were performed at least in triplicate, to test the reproducibility of the technic. Values within any one group agreed with each other in orders ranging from 2% to 12%. In tissues with very low levels of activity the percentage variations were greatest.

*Discussion.* Glycerophosphate oxidation is appreciable in all of the tissues studied although variations are encountered in terms of specific enzymes involved. The high levels of insoluble AGPO and AGPD in brain may be related to its high content of DPNase (6) since the insoluble enzymes are not DPN-dependent. Although only moderate increase in activity was elicited by addition of phenazine methosulfate and cyanide in kidney, liver, and brain, a striking increase (8-fold) was observed for the total enzyme

TABLE I. AGPO and AGPD Levels in Tissues of Mice.

| Organ | AGPO total | AGPO insoluble | AGPD total | AGPD insoluble |
|---|---|---|---|---|
| Kidney (11) | 6.3 ± .32* | 1.0 ± .13 | 8.9 ± .46 | 1.1 ± .19 |
| Liver (11) | 7.5 ± .49 | negligible | 8.1 ± .55 | negligible |
| Brain (11) | 5.0 ± .43 | 3.4 ± .30 | 5.5 ± .39 | 4.2 ± .32 |
| Muscle (11) | .8 ± .25 | negligible | 6.9 ± .86 | .4 ± .19 |

* Stand. error of mean.

of muscle. This may prehaps be explained by low levels of some flavoprotein moiety in muscle which is shunted in the assay for AGPD.

It has been reported(7) that female rat livers contain 4 times as much soluble AGPD as male rat livers, with both groups demonstrating extremely low activity. However, in a preliminary study in this laboratory with 3 female and 5 male rats (Sprague-Dawley), essentially similar mean values for soluble AGPD were found. These data [5.5 (5.2-5.9) in female and 5.4 (3.9-7.5) in male animals] are more consistent with usual findings of similarity of enzyme levels in the two sexes of the same species. The routine use of high levels of iodoacetate in the spectrophotometric method may account for the low levels of measurable AGPD activity described earlier.

*Summary.* AGP oxidation has been determined and characterized in kidney, liver, brain and muscle of the mouse. The assay technic utilized is simple, results are reproducible and only readily available laboratory equipment is required. In addition, enzyme inhibitors which might interfere with the accuracy of the determination are not used. It is felt that the present method may serve to broaden the limited lines of clinical investigation dealing with the significance of AGPD in regulation of metabolic processes.

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Received April 23, 1961. P.S.E.B.M., 1961, v107.

## Lipid Formation by Fibroblasts Cultured on Serum from Stressed Animals.[*]
(26676)

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Tissue cells cultivated *in vitro* are particularly sensitive to altered environment(1). An example is the tendency toward fat accumulation characteristic of most tissue culture cells, particularly those grown in presence of certain hormones(2). Preliminary investigations into possible physiological and morphological changes in tissue culture cells cultured on media containing serum from stressed chickens revealed an apparent alteration in cell physiology. Mouse fibroblasts were seen to accumulate lipid as intracytoplasmic droplets when cultured in the presence of serum from stressed chickens. This was not true in the presence of serum from non-stressed chickens or horses. The present paper constitutes a report on several experiments undertaken to establish and elaborate on the earlier preliminary observation.

*Materials and methods.* Young Leghorn cockerels were chosen as the test animals because of their availability, their notoriously labile emotional nature, and because of the ease with which their blood could be drawn. Early experiments revealed that hens could not be employed, evidently because of the toxicity of high serum calcium levels. Control animals were maintained in an isolated, enclosed, temperature-controlled (24 ± 2°C) incubator with subdued light. Care was taken to provide adequate bird space and proper pen flooring (to allow normal scratching), and to prevent disturbing influences by using indirect feeding and watering systems, in order to reach a basal level of external stress.

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[*] This research supported by a grant from Univ. of Nebraska Research Council. We wish gratefully to acknowledge the professional advice and assistance given by Drs. Donald Pace, I. William Brill and John L. Adams, Univ. of Nebraska.

Crowding(3) and heat provide 2 excellent forms of controlled stress for chickens, and these methods have been employed in the present studies. Test animals were maintained up to 8 days under (a) crowding conditions of 0.4 square foot to a bird—normal need is one square foot or more per 6 week old bird; and (b) conditions of elevated temperature, the test temperature being 41 ± 1°C.

With the birds in a fasting state,† serum was obtained by cardiac puncture at the end of each test period. This technic does not appear to disturb the chicken. A chemically defined medium was prepared containing 200 units penicillin, 0.2 mg streptomycin and 10% of this serum(4). After the filter sterilization, the medium was stored in pyrex glass tubes at approximately 5°C and used during the experiment.

Because of its apparently great sensitivity to environmental changes, the "Low-line" mouse fibroblast strain was used in most of the studies reported here(5). Other cell lines were used where noted. The experiments were carried out in Carrel flasks (10 flasks for each condition), using replicate culture technics(6). The flasks were inoculated with 1.5 million cells in 1 ml of the chemically defined medium plus 10% horse serum and incubated at 37°C in 5% $CO_2$-air to allow the cells to become established. After 2 days, this medium was replaced by 1 ml of medium containing 10% chicken serum, either control or test. Replacement was repeated every 2 days for the duration of experiment. Observations were made daily for growth and morphological changes, and the cells enumerated by means of the Coulter counter(7). From time to time, the cells were photographed.

Samples of each serum specimen were tested for total cholesterol by the method of Pearson, *et al.*(8) and for total fats by the Lipitest method(9). Sera were analyzed for ionic fatty acids (FFA, NEFA, UFA) by the method of Trout, *et al.*(10) further modified by the use of 0.02N KOH as titrant, and 0.1% thymolphthalein as the indicator.‡

*Results.* Serum from chickens subjected to stress by heating or crowding stimulates intracytoplasmic lipid droplet formation by "Low-line" mouse fibroblasts cultured on media containing that serum. Sera from non-stressed chickens do not elicit this lipid accumulation (Fig. 1). Growth curves of control *vs* "stressed" serum cultures (24, 48 and 96 hours stress) reveal that there is no appreciable variation in total cell counts (Fig. 2). Individual counts suggest a slight stimulation of growth by "stressed" sera at 24 hours which is not apparent in later counts. Droplets become apparent in 8 hours after addition of the "stressed" serum and while the cells are in the stage of active proliferations.§ They reach a maximum concentration in about 24 hours and maintain this maximum so long as the cells are in the presence of "stressed" serum. When "non-stressed" chicken or horse serum medium is substituted, the droplets slowly disappear and are gone by 6 days.

The phenomenon can be demonstrated easily in physiologically immature chickens but becomes difficult to demonstrate in adult chickens. Droplet formation is readily demonstrable in a 10% serum medium from a young cockerel, but a 30% serum medium is required to demonstrate comparable droplet formation in a rooster of 6 months or older. The phenomenon appears to be limited to mesenchymal cells of a non-malignant nature. Six cell lines in addition to the "Low-line" were studied in one experiment in which all were grown on the same pooled sera from 2 birds subjected to 24 hours of heat stress. The "Low-line" control again produced lipid

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† Ten to 12 hours after last feeding.

‡ These modifications were recommended by Dr. Herbert Davis, Dept. of Biochemistry, Univ. of Nebraska Coll. of Med.

§ Time-lapse cinematomicrography, not reported in detail here, reveals cellular division proceeding in presence of lipid droplets.

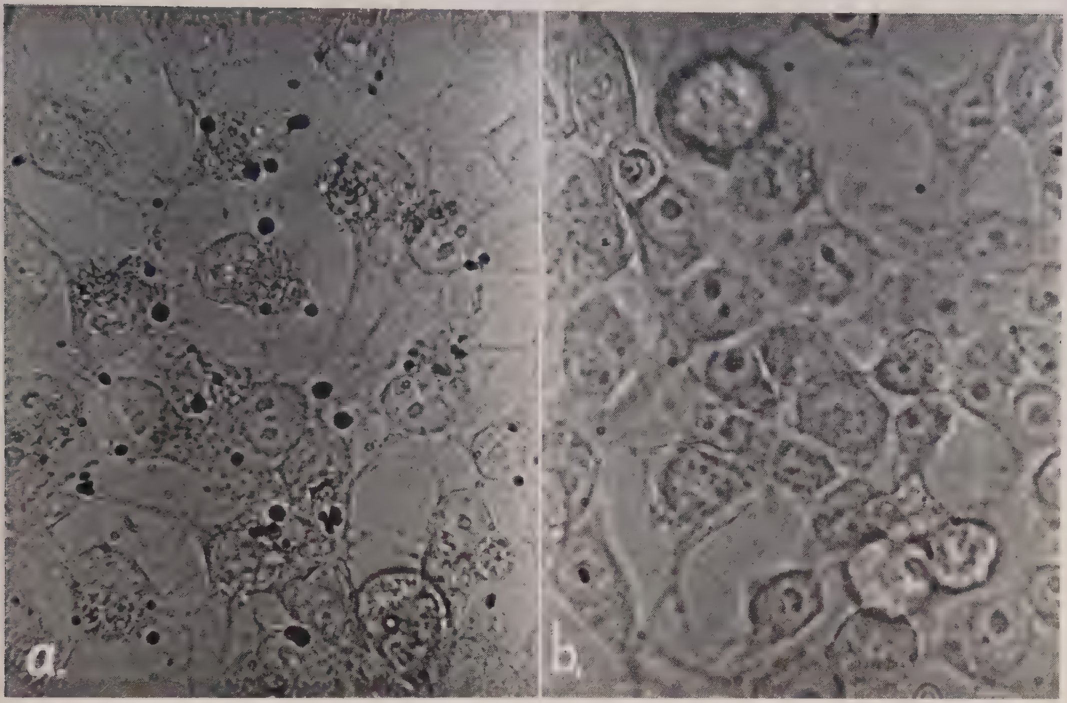

FIG. 1. Sudan III—stained cultures of "low-line" mouse fibroblasts grown on serum from (a) stressed and (b) non-stressed chickens. Approximately 650×.

droplets. Earle's L-strain produced a few. The malignant (High-line) mouse fibroblast did not produce droplets nor did the epithelial or endothelial lines (HeLa, mouse liver, human lung and human skin).

Stress-induced elevation of various serum lipid constituents is well known(11,12), as is the role of pituitary-adrenal hormones in lipogenesis(13,14). It seemed important, therefore, to ascertain whether an increase in available serum lipid might not explain the observed accumulation of lipid. Twenty-eight different sera were analyzed for total lipid and cholesterol (Table I). There appeared to be no relation between the serum lipid levels and development or non-development of intracytoplasmic droplets. As an

TABLE I. Serum Cholesterol and Total Lipid Levels in Stressed and Non-Stressed Chickens.

| | | Serum cholesterol (mg %) | |
|---|---|---|---|
| | No. | Range | Avg |
| Non-stressed controls* | 10 | 108-146 | 126 |
| 24-hr stressed† | 9 | 98-195 | 136 |
| 96-hr " † | 4 | 98-190 | 155 |
| Tranquilized, controls‡ | 3 | 130-132 | 131 |
| " , 24-hr stress‡ | 2 | 130-178 | 154 |
| | | Total lipids (mg %) | |
| Non-stressed controls* | 7 | 227-402 | 290 |
| 24-hr stressed† | 7 | 157-376 | 240 |
| 96-hr " † | 4 | 219-315 | 277 |
| Tranquilized, controls‡ | 3 | 175-310 | 237 |
| " , 24-hr stress‡ | 2 | 157-236 | 196 |

* All control samples produced no droplets in cell culture.

† All test samples produced droplets in cell culture.

‡ Reserpine added to food in concentrations of 5 mg %.

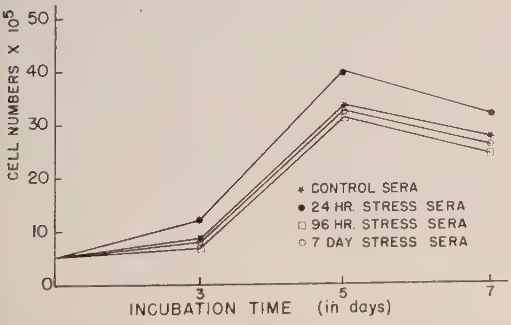

FIG. 2. Growth of mouse fibroblasts on media containing "non-stressed" and "stressed" chicken sera.

TABLE II. Serum Ionic Fatty Acids in Stressed and Non-Stressed Chickens.

| | No. | Serum ionic fatty acids (meq/l) Range | Avg |
|---|---|---|---|
| Non-stressed controls | 6 | .297-.813 | .484* |
| Stressed (3 to 24 hr) | 9 | .270-.861 | .564* |
| Horse serum | 4 | .559-.608 | .548 |

* Difference between non-stressed and stressed serum averages, calculated as sodium oleate, is 2.4 mg %.

example, some stressed sera contained 98 mg% cholesterol and produced droplets, while some non-stressed sera contained as much as 146 mg% and did not produce droplets.

Simms(15) has reported lipid "granule" formation by fibroblasts cultured on media containing added fatty acid soaps. Similar observations in our laboratory led us to consider the possibility that increased serum fatty acid levels might be the source of the accumulated lipid.‖ However, a series of ionic fatty acid determinations on stressed and non-stressed chicken sera and on horse sera (which does not stimulate droplet formation under the conditions of our experiments) indicate that the level of free fatty acids is not a significant factor in their production (Table II). Most of the sera tested contained less than 20 mg% (0.66 meq/l) of free fatty acid when calculated as sodium oleate. Grossfield(2) arrived at a similar conclusion while studying lipid vacuole formation by tissue culture cells, reporting that "The lipid appears predominantly to be produced by the cell and not to come from the medium."

*Discussion.* The results presented here strongly suggest a humoral mechanism for production of a substance eliciting the observed phenomenon of lipid accumulation by fibroblasts cultured on serum from chickens subjected to crowding and heat stress. That the test cell used is a cloned fibroblast and is not, *per se*, a lipocyte, indicates that this is not a usual function of this cell and might, therefore, be considered abnormal. That lipid droplet production occurs during active cell growth and multiplication and not during a period of cultural decay and cell death suggests that the process is not one of degeneration. The apparent limitation of the phenomenon to cells of mesenchymal origin is not without precedent, Kline, *et al.*(16) having reported similar observations with hydrocortisone stimulation of various cell lines.

The relationship between physiological youth and production of this stress factor is interesting in that Weiss(17) has recently reported similar observations, wherein he noted acclimatization to cold was best achieved by the relatively young rat. In this regard also, the frequently observed lipogenic effect of the growth hormone from the anterior pituitary(18) and production of a lipid mobilizer from the anterior pituitary by stress(19) are extremely interesting. Whether all of these widely diverse observations surrounding lipid mobilization by physiologically young animals can be equated with Holman's theory of first stage atherogenesis in humans by "fatty streaking," which occurs between the ages of 0 to 19 years of age(20), remains to be seen, but it appears worthy of consideration.

*Summary.* Certain tissue cells, grown *in vitro* on media containing serum from stressed chickens, produce quantities of lipid deposited in droplets within their cytoplasm. Serum from non-stressed animals does not elicit this phenomenon. Being associated with a period of active proliferation, it cannot be said to be a degenerative process. Evidently it is not related to increased lipid content of the serum. The stress factor, if such there be, is more easily elicitable during physiological youth of the experimental animal. It is suggested that the phenomenon is humoral stimulated and may be a manifestation, *in vitro* of elevated circulating stress hormones *in vivo*.

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‖ It was determined that 20 mg% or over of sodium oleate was required to produce droplets similar to those observed on "stressed" serum. A level of 35 mg% or over was found to be cytotoxic.

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Received April 24, 1961. P.S.E.B.M., 1961, v107.

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# Modification of Lethal Effect of Bacterial Endotoxin by Substances Altering the Metabolism of 5-Hydroxytryptamine.* (26677)

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(Introduced by P. Reznikoff)
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5-Hydroxytryptamine (5HT), which in free form is a potent stimulator of smooth muscle, normally circulates bound to platelets in an inactive state(1). The occurrence of thrombocytopenia and elevated plasma concentrations of 5HT after intravenous injection of endotoxins from Gram-negative bacteria(2-3) suggests that some of the biologic effects of endotoxins might be related to release of 5 HT from damaged or sequestered platelets. The present studies were undertaken to determine the effect of substances that alter the metabolism of 5HT on the lethal action of bacterial endotoxin in mice.

*Materials and methods.* Three substances that alter 5HT metabolism were investigated: (A) 1-benzyl-2, 5-dimethyl serotonin (BAS),‡ an antimetabolite of 5HT by virtue of similar chemical configuration(4), (B) beta-phenyl isopropyl hydrazine (PIH)§(5), an inhibitor of monamine oxidase which catalyzes the degradation of 5HT to 5-hydroxy indole acetic acid(6), and (C) the metabolic precursor of 5HT, 5 hydroxy tryptophan (5-HTP)‖, administration of which appreciably increases tissue stores and platelet concentrations of 5HT(7). Dosages of BAS, PIH, and 5HTP were based on the reports of Shaw and Wooley(4), Horita(5), and Udenfriend *et al.*(7), and were somewhat larger than necessary for physiologic activity. These substances produced no apparent untoward effects when administered alone to control groups of 10 or more mice.

Eighteen to 25 g male albino mice of the CFW strain (Carworth Farms, New City, N. Y.) were used in all experiments. Groups of 10-20 mice were injected by the intraperitoneal (IP) or subcutaneous (SC) route with the substance to be tested on each of 2 suc-

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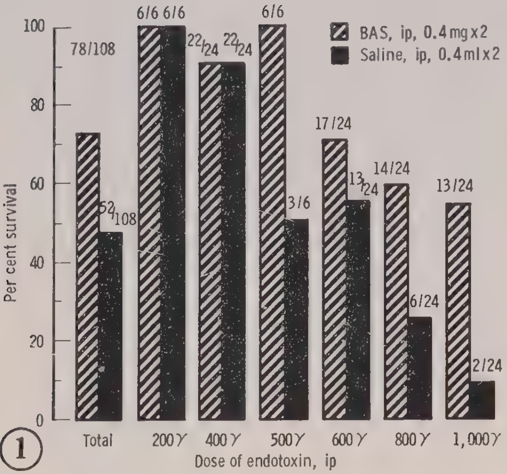
* This study supported by USPHS grant.

† Edward Livingston Trudeau Fellow of American Thoracic Society.

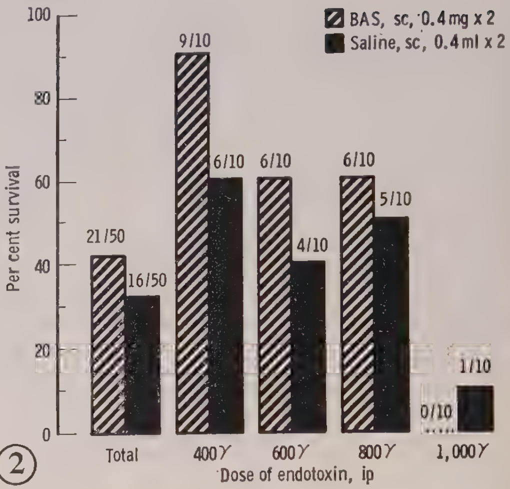
‡ Merck, Sharp, and Dohme Research Laboratories, Rahway, N. J.

§ Lakeside Laboratories, Milwaukee, Wis.

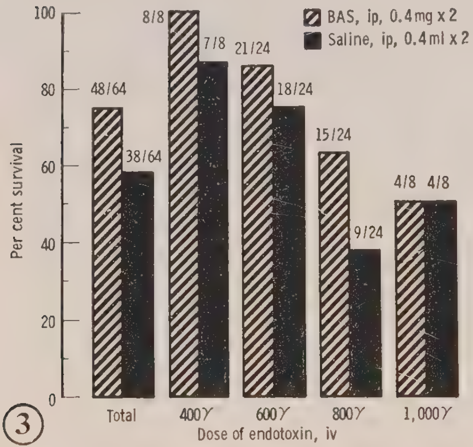
‖ Nutritional Biochemicals, Cleveland, Ohio.



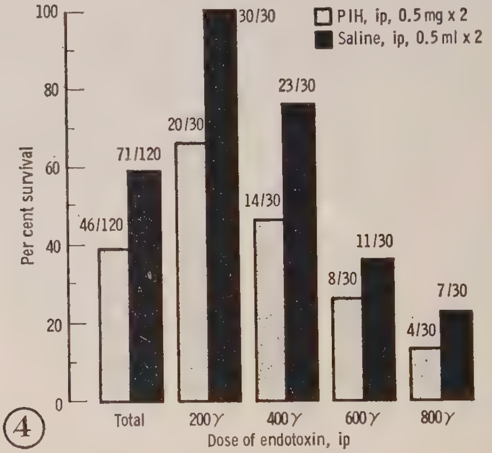
① P = .015 (total only)



② P >0.2 (total only)



③ P = .070 (total only)



④ P = .002 (total only)

FIG. 1. Effect of BAS inj. IP on response of mice to IP challenge with endotoxin.
FIG. 2. Effect of BAS inj. SC on response of mice to IP challenge with endotoxin.
FIG. 3. Effect of BAS inj. IP on response of mice to IV challenge with endotoxin.
FIG. 4. Effect of PIH inj. IP on response of mice to IP challenge with endotoxin.

cessive days. Controls were injected with normal saline solution. Both pretreated and control groups were then challenged with graded doses of *Escherichia coli* endotoxin (Difco, Lot No. 0127:B8) by the IP or intravenous (IV) route 30-90 minutes after the last pretreatment injection. Mortality was tabulated after 24 and 48 hours. For statistical purposes the groups receiving different doses of endotoxin were combined and the difference in per cent survival between pretreated and control groups submitted to the t test.

*Results.* As shown in Fig. 1 mice pretreated with BAS by the IP route were significantly more resistant than saline pretreated controls to the lethal action of endotoxin injected by the same route. When either BAS or endotoxin were administered by other than the IP route (Fig. 2 and 3) a protective effect was observed but was less marked and not significant according to this method of statistical analysis. Fig. 4 illustrates that pretreatment with the monoamine oxidase inhibitor PIH rendered mice significantly more susceptible to the lethal action

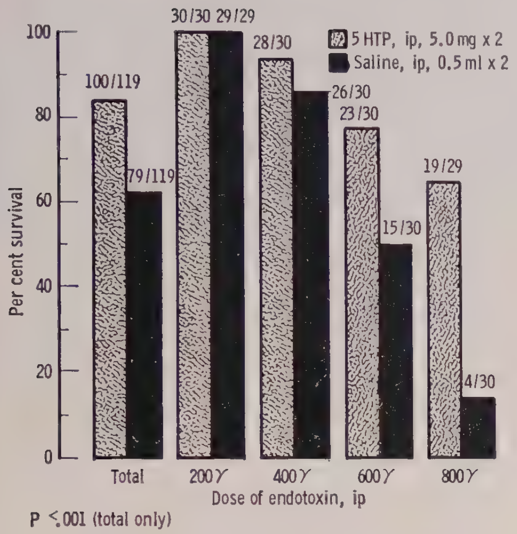


FIG. 5. Effect of 5HTP inj. IP on response of mice to IP challenge with endotoxin.

of endotoxin than saline pretreated controls. Contrary to expectations, pretreatment with the 5HT precursor, 5HTP, significantly enhanced the ability of mice to survive subsequently administered endotoxin injections (Fig. 5).

*Discussion.* Inhibition of the lethal effect of endotoxin in mice by pretreatment with the anti-5HT compound BAS, and its enhancement by pretreatment with the monoamine oxidase inhibitor PIH suggest that release of 5HT may be a factor in endotoxin lethality. It should be emphasized that these changes were only partial and were influenced by route of administration of both pretreatment and challenge injections.

Shimamoto and associates(8) have reported similar results in rabbits treated with the anti-5HT compounds Brom-lysergic acid diethylamid (BOL) and the monoamine oxidase inhibitor iproniazid. Gilbert(9) reported that the anti-5HT compound lysergic acid diethylamid (LSD) mitigated the acute hypotensive response observed in cats after intravenous injections of endotoxin but in a single instance found oral BAS without effect. Several groups of investigators(10,11, 12,13) have demonstrated that chlorpromazine inhibits the lethal effect of endotoxin, a property possibly attributable in part to its anti-5HT activity. Contrary to our findings, two groups of investigators(11,14) have reported that monoamine oxidase inhibitors are without effect on endotoxin lethality.

The protective effect of 5HTP, the metabolic precursor of 5HT, was not anticipated. However, Gordon and Lipton(15), and Lasker *et al.*(11) observed that mice pretreated with 5HT itself demonstrated enhanced resistance to endotoxin. Although the effects of pretreatment with 5HTP and 5HT are superficially discordant with those described for BAS and PIH, these interrelationships are probably too complex to warrant the assumption that they are contradictory. A similarly confusing situation exists in the case of histamine, another amine with marked physiologic activity which is normally bound to platelets and known to be released after injection of endotoxin(16). Pretreatment with this substance also diminishes the lethality of subsequently administered endotoxin(10,11), as does pretreatment with histamine depletors(17) and antihistaminic compounds(10,11).

*Summary.* The lethal effect of bacterial endotoxin in mice is modified by administration of the 5HT antagonist, BAS, the monoamine oxidase inhibitor PIH, or the 5HT precursor, 5 HTP. Animals pretreated with BAS and 5 HTP demonstrated increased resistance to endotoxin whereas pretreatment with PIH increased susceptibility to endotoxin.

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Received April 24, 1961. P.S.E.B.M., 1961, v107.

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# Alkaline Phosphatase Activity of Human Cell Cultures.* (26678)

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Studies of enzyme activities, nutritional requirements and metabolic activities associated with growth of mammalian cells in culture have in general revealed basically similar patterns regardless of the source from which those cells had been derived(1,2). During studies to characterize the tissue of origin of established human cell cultures, differences in alkaline phosphomonoesterase activity were observed(3). These observations have been extended to other cell lines, including recent isolates, and this report which describes differences in phosphatase activity between clonal strains of several parental lines, reemphasizes the heterogeneity which may exist in cell populations propagated *in vitro*.

*Materials and methods.* Established cell lines were purchased from Microbiological Associates, Inc., and clonal strains HeLa $S_3$ and 3213A were provided by Drs. T. T. Puck and M. Bender respectively. The parental Zimmer liver line was derived from a biopsy in a 5 month old infant with congenital atresia of the bile ducts and has been maintained in serial culture for approximately 70 passages. Recent cell isolates were derived according to standard technics from bone marrow aspiration(4) and skin biopsy(5). Clonal strains were derived from one or more consecutive single cell platings according to the procedure of Puck *et al.*(6).

Eagle's medium and M 199 containing 10 or 15% undialyzed human, horse or calf serum or the nutrient medium of Puck containing 20% undialyzed human serum were used for the growth of established cell lines. Recent isolates were cultured in an enriched Eagle's medium with added bovine embryo extract ultrafiltrate(4). After growth for 6-8 days on glass surfaces in an atmosphere of 5% $CO_2$ in air at 37°C, cells were dispersed with .05% trypsin in bicarbonate-free salt solution(7), and transferred to fresh medium, or harvested by centrifugation at 500 × g at room temperature after neutralization of tryptic action with growth medium. The cells were washed twice in 20 volumes of cold 0.14 M NaCl or 0.25 M sucrose and resuspended in 0.25 M sucrose. Cell-free extracts were prepared by sonic vibration of 2 to 5 ml of cell suspensions in collodion-treated lusteroid tubes for 1 to 5 minutes in an ice water-cooled Raytheon 10 kc oscillator.

Alkaline phosphatase was measured by hydrolysis of disodium p-nitrophenyl phosphate using 2-amino-2-methyl-1-propanol-HCl (AMP) buffer at pH 10.6 and 38°C(8). Specific activity was expressed as $\mu$M of p-nitrophenol liberated in 30 minutes per mg of cell protein(9).

*Results.* Table I summarizes the specific activities for alkaline phosphatase of a variety of established and recently isolated human cell lines. Each value represents the

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* This investigation was supported in part by grants from Nat. Inst. of Arthritis and Metab. Dis., P.H.S., National Assn. for Retarded Children, and Assn. for the Aid of Crippled Children.

TABLE I. Alkaline Phosphatase Activity of Human Cell Cultures.

| Established cell cultures | Specific activity* | Recent cell isolates | Specific activity |
|---|---|---|---|
| Detroit-6 | 170.7 | Pe. marrow | 90.9 |
| J-111 | 127.2 | As. marrow | 29.3 |
| Conjunctiva | 106.0 | Tu. skin | 12.5 |
| H.Ep. #2 | 96.5 | He. marrow | 8.7 |
| Heart | 51.2 | Ch. skin | 2.4 |
| Chang liver | 25.8 | | |
| KB | 7.9 | | |
| Intestine | 6.1 | | |
| Zi. liver | 1.1 | | |
| HeLa S-3 | 0.3 | | |

* Mean specific activity = $\mu$M p-nitrophenyl phosphate hydrolyzed per mg protein in 30 min. at 38°C in AMP buffer, pH 10.6.

mean of activities at termination of at least 4 consecutive 6-8 day growth cycles. There is a 500-fold difference between the phosphatase activities of established cell cultures; the highest values observed were those with the Detroit-6 and J-111 lines. Recent isolates from skin biopsy and bone marrow aspiration also showed a wide range of phosphatase activity even during the initial 4 or 5 passages *in vitro*. The variation in activity between different cell lines showed no relation to cellular morphology, nor did propagation in a particular nutrient medium explain the differences observed, since cell lines with high and low activity were found under the same growth conditions.

TABLE II. Changes in Alkaline Phosphatase Activity of Chang Liver during Serial Transfer Following Change of Nutrient Medium.

| Days following medium alteration | Medium | Activity* |
|---|---|---|
| Initial | Eagle's + 10% horse serum | 51.0† |
| 7 | Puck's + 20% human serum | 54.8 |
| 19 | *Idem* | 14.9 |
| 25 | " | 8.4 |
| 33 | " | 11.4 |
| 40 | " | 12.1 |
| 47 | " | 15.6 |
| 55 | " | 39.0 |
| 77 | " | 70.1 |
| 99 | " | 72.3 |
| 107 | " | 87.4 |

* Specific activity = $\mu$M p-nitrophenyl phosphate hydrolyzed/mg protein in 30 min. at 38°C in AMP buffer, pH 10.6.

† Mean activity of cell preparations from 4 consecutive weekly growth cycles prior to transfer to new medium (individual values = 44.9, 41.0, 62.4, 55.7).

On the other hand, changes in alkaline phosphatase activity for a particular cell line were occasionally observed following alteration of the nutrient environment, as is shown in Table II with Chang liver cells following transfer from Eagle's medium containing horse serum to Puck's medium with human serum. This suggested the possibility that diverse cell types existed in the population, one or more of which might find a selective advantage in the new metabolic situation and so outgrow the other cells.

In support of this concept were the findings with clonal strains of various parental cell lines. Fig. 1 shows the distribution of individual values and mean specific activities of alkaline phosphatase for clonal strains of Chang liver and Zimmer liver and subclones of HeLa $S_3$, during serial transfer in Puck's medium for 20 or more passages. There is a considerable difference in activity between the clonal strains. For example, Clone 4 of Chang liver, which can be distinguished from other Chang strains by the morphological characteristic of a smaller cross sectional area, has an average specific activity which is only 1/250th that of Clones 8A or 9. Similarly, a 6-fold difference in mean phospha-

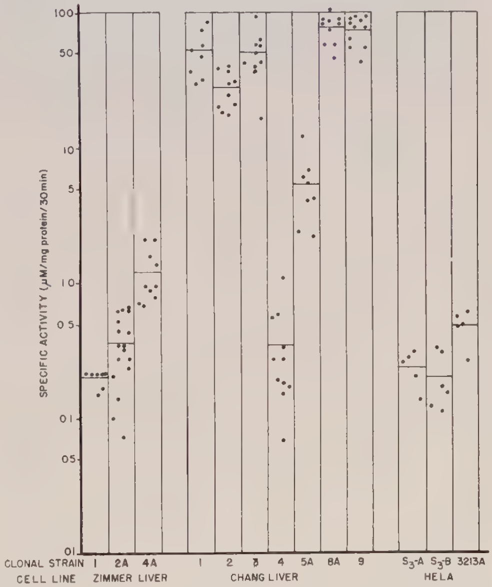

FIG. 1. Distribution of specific activities of alkaline phosphatase for clonal strains of various cell lines.

tase activity is observed between Clone 1 and 4A of Zimmer liver, with no overlap between the maximum range of values. On the other hand, phosphatase activities of subclones A and B of HeLa $S_3$ are similar, and slightly lower than that of clonal strain HeLa 3213A.

*Discussion.* Although there is general uniformity in nutritional and metabolic characteristics, individual differences have been observed among certain cell lines in culture(1). The striking difference in alkaline phosphomonoesterase activity which has been noted also by Cox and MacLeod(10) is another such finding. Similar results have been obtained with histochemical technics(11,12,13) although these methods do not lend themselves to precise quantitation. Kovacs also has reported differences in phosphatase activity as measured at pH 7.6 with a biochemical assay using intact cell cultures(14).

The phosphatase activity of sonicates of a given cell line remains constant during serial propagation under uniform environmental conditions. However, the variability occasionally observed following transfer to a different nutrient medium suggested the existence of diverse cell types in the population, one or more of which might find a selective advantage as a result of the new metabolic situation and so outgrow the other cells. Studies with clonal cell strains derived from several parental lines showed significant differences in alkaline phosphatase activity, in support of this concept. In fact, the maximum range of activity with clonal isolates from a single parental line was almost as great as the range of activity with different parental lines. These differences are in agreement with the findings that clonal strains may have distinct physiological and morphological properties as well as observable differences in their karyotype(15,16). Since the available evidence suggests that the phenotypic variability of aneuploid cell populations may be caused by changes in the chromosomal constitutions of cells(16) it will be of interest to determine whether alterations of karyotype can be correlated with differences in alkaline phosphatase activity of various clonal strains.

*Summary.* Studies of alkaline phosphatase activity of sonicates of human cell cultures have revealed striking quantitative differences between established and recently isolated cell lines of diverse origin. Marked differences also have been noted between clonal strains derived from one or more consecutive single cell platings from several cell lines. The changes in phosphatase activity during serial passage following alteration of the nutrient medium may have resulted from a change in the relative distribution of cell types within the parental population.

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Received April 25, 1961. P.S.E.B.M., 1961, v107.

# Response of Vitamin $B_6$-Deficient Rats to Non-Hypophyseal Gonadotrophins.[*] (26679)

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Previous studies have shown the consistent occurrence of reproductive disturbance in adult female vitamin $B_6$-deficient rats(1,2). Reproductive failure was apparently due to lack of ovarian hormones as pregnancy could be maintained in 90% of such rats by daily injection of estrone and progesterone(3) at dosage levels known to be effective in the hypophysectomized-oophorectomized rat(4). Injection of pituitary gonadotrophins was, however, less successful in maintaining pregnancy in $B_6$-deficient rats, a finding which suggests the possibility of a decreased ovarian response to these hormones(5). Ovarian response in virgin $B_6$-deficient rats to injected hypophyseal follicle-stimulating hormone (FSH) was markedly decreased when compared with the response obtained in adult hypophysectomized rats, whereas only a slightly decreased response to hypophyseal interstitial-cell-stimulating hormone (ICSH) was observed in $B_6$-deficient rats(6). The present report[‡] describes the response of virgin $B_6$-deficient rats as compared with normal immature rats to graded levels of 2 non-hypophyseal gonadotrophins, human chorionic gonadotrophin (HCG) and equine gonadotrophin (PMS).

*Methods.* Normal female rats (Long-Evans strain) were given the Vit. $B_6$-deficient diet[§] when 80 to 85 days of age. Vaginal smears were examined daily and the rats weighed and their condition described every 5 days. After 60 to 65 days on the diet, those $B_6$-deficient animals which showed definite signs of deficiency, *e.g.*, cessation of growth and progressive acrodynia, were distributed into equivalent groups on the basis of body weight and length of anestrus. The rats had been anestrous for an average of 44 days and had lost approximately 10% of their body weight. Varying levels of HCG[‖] were injected subcutaneously once daily for 4 successive days; PMS[‖] was given in a single subcutaneous dose. Normal immature rats, 24 days of age and maintained on a stock diet of natural foodstuffs,[¶] were injected with the same levels of these hormones as $B_6$-deficient animals. All dosage levels were repeated at least once for both types of animals. Normal immature rather than hypophysectomized rats served as controls in this study, since hypophysectomized rats are less responsive to low levels of HCG and PMS(9). All rats were autopsied 96 hours

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[*] Aided by grants from USPHS and Roche Anniversary Foundation. We are greatly indebted to Dr. Randolph Major, Merck and Co., Rahway, N. J., for generous supplies of deoxypyridoxine, 2-methyl-1, 4-naphthoquinone, and crystalline B vitamins; to Dr. Elmer L. Severinghaus, Hoffmann-La Roche, Nutley, N. J., for crystalline *d*-biotin, *d*-calcium pantothenate, and *dl*-alphatocopherol; and Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., for synthetic pteroylglutamic acid.

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[‡] A preliminary report of this study was made before Am. Inst. of Nutrition(7).

[§] The vit. $B_6$-deficient diet(3,5,6) consisted of 24% alcohol extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primax), 4% salts No. 4 and 0.5 mg% deoxypyridoxine, the vit. $B_6$ antimetabolite. Crystalline vitamins per kilo of diet were 300 µg *d*-biotin, 5 mg 2-methyl-1, 4-naphthoquinone, 5 mg thiamine HCl, 5.5 mg pteroylglutamic acid, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg niacin, 50 mg *d*-calcium pantothenate, 400 mg inositol, and 1.0 g choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 800 USP units vit A, 115 chick units vit D, 6 mg synthetic *dl*-alphatocopherol, and 650 mg corn oil (Mazola).

[‖] HCG ("Antophysin") was prepared by Winthrop Laboratories and PMS ("Gonadin") by Cutter Laboratories.

[¶] Composition of this stock diet (XIV) is given in Srebnik *et al.*(8).

TABLE I. Response to HCG in Vit. $B_6$-Deficient and in Normal Immature Rats.

| Total dose, IU | No. of rats | Ovaries: Wt, mg | Ovaries: Follicles* | Ovaries: Interstitial cells | Uterine wt, mg |
|---|---|---|---|---|---|
| | | | Vit. $B_6$-deficient | | |
| 0 | 22 | 34 ± 2† | sm, m | Atrophic | 109 ± 5 |
| 1.25 | 6 | 31 ± 3 | " " | " | 117 ± 8 |
| 2.5 | 8 | 28 ± 9 | sm, m, few ml-l | " | 189 ± 21 |
| 5.0 | 8 | 48 ± 5 | m-l, CL | Repair | 240 ± 37 |
| | | | Normal immature | | |
| 0 | 10 | 19 ± 1 | sm, few m | Epithelioid | 22 ± 1 |
| 1.25 | 10 | 18 ± 2 | " " | " | 23 ± 2 |
| 2.5 | 10 | 18 ± 1 | sm, m, ml-l | " | 116 ± 18 |
| 5.0 | 5 | 28 ± 3 | few ml-l, CL | " | 102 ± 6 |

* Follicles were measured with an ocular micrometer and designated as small (s) = 375 μ, small-medium (sm) = 450-500 μ, medium (m) = 550-600 μ, medium-large (ml) = 700-750 μ, and large (l) = 800-1000 μ. The presence of new corpora lutea (CL) was recorded.

† Stand. error of mean.

after first injection. Ovaries and uteri were weighed at autopsy. The ovaries were fixed in Bouin's fluid, embedded in nitrocellulose, serially sectioned, and stained with hematoxylin and eosin. Criteria for ovarian responses were those used in previous studies (6,8). Follicle-stimulating activity was detected by an increase in size and number of ovarian follicles as compared with those in uninjected controls, and interstitial-cell-stimulating activity by a histologically detectable response designated as partial repair of the atrophic interstitial cells(10). Additional criteria were corpus luteum formation, significant increase in uterine weights, estrous vaginal smears in adult animals and vaginal openings in immature rats.

*Results.* The degree of follicular development was approximately the same in the ovaries of $B_6$-deficient and normal immature rats before hormone injections were started; both contained small-medium and a few medium-sized follicles. In uninjected $B_6$-deficient animals the ovarian interstitial cells were atrophic with pyknotic nuclei and degenerating, pale-staining cytoplasm; residual corpora lutea similar to those observed after hypophysectomy were present. No corpora lutea were present in the ovaries of normal immature rats and the interstitial tissue was epithelioid.

Vit. $B_6$-deficient rats responded to the same dosage levels of HCG as normal immature rats; follicular maturation and increased uterine weights occurred in both groups when 2.5 IU were given (Table I). Injection of 5 IU caused corpus luteum formation in both groups and produced vaginal opening in immature rats and estrous vaginal smears in $B_6$-deficient animals. In addition, the atrophic interstitial tissue was stimulated in $B_6$-deficient rats with this dosage level.

Vit. $B_6$-deficient rats were somewhat less sensitive to PMS than normal immature rats (Table II). Follicular development and increases in uterine weight were observed in some normal immature rats when 0.5 IU were given whereas $B_6$-deficient animals required one IU for this effect. Corpus luteum formation occurred in all normal immature rats injected with one IU but $B_6$-deficient rats needed 2 IU for this response. Partial repair of interstitial tissue and vaginal estrus were also observed in $B_6$-deficient animals given 2 IU. Higher dosage levels (5-10 IU) significantly increased the number of follicles and corpora lutea in normal immature rats but not in $B_6$-deficient animals.

*Discussion.* This study has shown that Vit. $B_6$-deficient rats respond to low levels of the non-hypophyseal gonadotrophins, although they required slightly higher dosages of PMS than normal immature rats for equivalent ovarian effects. These low levels of HCG and PMS which cause stimulation of follicular growth in $B_6$-deficient rats may be contrasted with the high levels of exogenous hypophyseal FSH required for the same effect. Wooten *et al.*(6) have shown that 16

TABLE II. Response to PMS in Vit. $B_6$-Deficient and in Normal Immature Rats.

| Total dose, IU | No. of rats | Ovaries Wt, mg | Ovaries Follicles | Ovaries Interstitial cells | Uterine wt, mg |
|---|---|---|---|---|---|
| | | | Vit. $B_6$-deficient | | |
| 0 | 24 | 34 ± 2 | sm, m | Atrophic | 105 ± 4 |
| .5 | 8 | 33 ± 4 | " " | " | 120 ± 14 |
| 1 | 12 | 32 ± 3 | m, few ml-l | " | 187 ± 22 |
| 2 | 9 | 43 ± 2 | ", few ml-l, CL | Partial repair | 235 ± 19 |
| 5 | 8 | 50 ± 5 | " " " " | " " | 221 ± 19 |
| 10 | 8 | 52 ± 3 | " " " " | Repair | 196 ± 15 |
| | | | Normal immature | | |
| 0 | 19 | 17 ± 1 | sm, few m | Epithelioid | 27 ± 2 |
| .5 | 14 | 16 ± 1 | m, few ml-l | " | 63 ± 12 |
| 1 | 15 | 25 ± 1 | few m-ml, CL | " | 110 ± 3 |
| 2 | 13 | 26 ± 1 | " " | " | 81 ± 8 |
| 5 | 9 | 39 ± 7 | many m-ml, CL | " | 73 ± 7 |
| 10 | 9 | 56 ± 11 | " " | " | 84 ± 12 |

Abbreviations as in Table I.

RU of injected hypophyseal FSH were needed for follicular stimulation in $B_6$-deficient rats as compared with 2 RU for normal immature or adult hypophysectomized animals. It is generally assumed that the follicle-stimulating effect of low levels of HCG and PMS is due to release of FSH from the animal's own pituitary, the hormone then synergizing with the ICSH-like activity of the injected non-pituitary gonadotrophins (9). Since the pituitaries of $B_6$-deficient rats contain considerable FSH(11) this may have been released following injection of HCG and PMS. This study suggests that $B_6$-deficiency does not affect the ovarian response to endogenous FSH and that the observed insensitivity in this deficiency to exogenous hypophyseal FSH preparations may be of different origin.

The only comparable study of response to these gonadotrophins(8) has shown that rats deprived of dietary protein for one month responded to lower levels of HCG and PMS than did normal immature rats. The factors responsible for the greater effectiveness of these non-hypophyseal gonadotrophins in protein-deficient rats are apparently not operative in $B_6$-deficient animals.

*Summary*. The response of Vit. $B_6$-deficient rats to graded doses of 2 non-hypophyseal gonadotrophins, HCG and PMS, was determined and compared with that of normal immature rats. Follicular growth, corpus luteum formation, and increased uterine weights occurred with the same levels of HCG in both $B_6$-deficient and normal immature rats whereas slightly more PMS was required by the $B_6$-deficient than the normal immature rat to obtain an equivalent ovarian response.

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Received April 26, 1961. P.S.E.B.M., 1961, v107.

# The Validity of Using Plasma Corticosterone as a Measure of Stress in the Turkey.*†‡ (26680)

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The present emphasis on stress in relation to disease has resulted in a tendency to classify as stress factors many of the unfavorable environmental and management factors of poultry, without first obtaining evidence of measured changes in the function of the adrenal glands.

Identification of adrenocortical steroids has been accomplished only recently in birds. The adrenal effluent blood of the capon has been reported to contain corticosterone, cortisone, and aldosterone(1), of which corticosterone was the principal component. Incubation of White Leghorn cockerel adrenals in Krebs-Ringer bicarbonate led to identification of corticosterone, aldosterone and tentative identification of small amounts of hydrocortisone(2). Corticosterone was the principal steroid obtained. Plasma levels of corticosterone in Gallinaceous birds have been reported. Adrenocorticotrophic hormone (8 I.U. ACTH intravenously) significantly increased corticosterone in adrenal effluent blood in pheasants and chickens 60 minutes after injection(3).

The present paper attempts to confirm that corticosterone is the principal corticosteroid found in turkey plasma. Data are also presented in support of the hypothesis that, *the concentration of corticosterone in peripheral plasma reflects the severity of stress conditions in the fowl.*

*Materials and methods.* Small White turkey poults were raised in batteries and were fed the Ohio Turkey Starter Ration(4) to 6 weeks of age for use in all experiments.

The procedure used to identify corticosterone was as follows: A 500 ml sample of plasma was extracted 3 times with 2 volumes of ethyl acetate, washed once with 0.1 volume of N NaOH and twice with water. The extract was dried by adding excess anhydrous $Na_2SO_4$ followed by filtration under vacuum through a cake of $Na_2SO_4$. This extract was then taken to dryness under vacuum in a water bath at 40°C. The residue was taken up in a small volume of ethyl acetate and layered onto a silica gel column(5). The column was first washed with a small amount of petroleum ether (B.P., 30-60°C) followed by 5% petroleum ether in benzene. The adrenal steroids were then eluted with ethyl acetate. The eluate was taken to dryness under vacuum in a water bath at 40°C. The residue was transferred in methanol to washed Whatman #1 filter paper and chromatographed with standards on a parallel lane in a system of ethylene glycol/toluene (6). The areas corresponding to the steroid standards were eluted in ethanol and rechromatographed in the $E_2B$ system(7).

Identification of corticosterone was based on (1) mobility with reference to standards run on a parallel lane in the 2 solvent systems; (2) reaction with diphenyltetrazolium chloride and (3) sulfuric acid spectrum(8).

The level of free corticosterone in extracts of aliquots of peripheral (2 ml) plasma was determined by a fluorescence technic(9). Fluorescence was read in a Coleman Model 12 photofluorometer. Although this method measures hydrocorticosterone as well as corticosterone, the fluorescence attributable to the former compound is negligible since only trace amounts of it can be detected by chromatographic technics.

*Results and discussion. Identification of corticosterone.* A large number of chromatograms was used in development of the method. The results are summarized diagramatically in Fig. 1. The steroid present

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* Approved for publication on 4-13-61 as Journal Article No. 53-61 by Associate Director, Ohio Agric. Exp. Station.

† This investigation was conducted as part of the North Central Regional Project, (Physiological Responses of Laying Fowl to Their Environment).

‡ Gratitude is expressed to the Upjohn Company for supplying the crystalline corticosteroids used in this study.

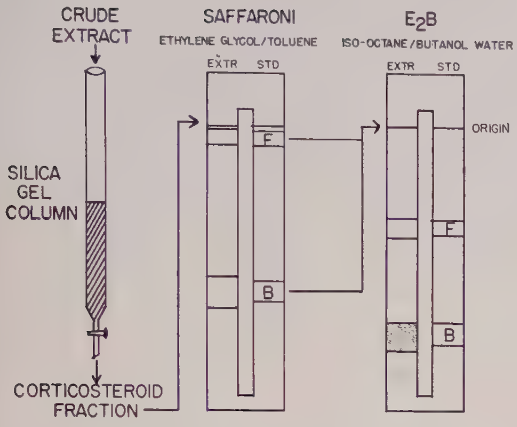

FIG. 1. Chromatographic separation of corticosterone (Compound B) and hydrocortisone (Compound F) from 200 ml equivalent of turkey plasma. Density of stippling indicates relative amounts of steroid.

in the largest quantity was identified as corticosterone. The material moved with a polarity of standard corticosterone run in parallel in both solvent systems, and reacted strongly with diphenyltetrazolium chloride. The sulfuric acid spectrum was substantially the same as that of pure crystalline corticosterone with the major peak at 285 mμ and smaller peaks at 333 mμ, 374 mμ, and 455 mμ. Ratio of corticosterone to hydrocortisone was approximately 20:1.

*Measurement of corticosterone in normal and hypophysectomized turkeys subjected to ACTH injections and various stressors.* The establishment of corticosterone as the predominant free corticosteroid found in turkey plasma suggests that concentration of this compound should reflect the severity of stress conditions in the fowl. Before this can be accepted as a working hypothesis, however, it must be shown: (1) that exogenous adrenocorticotrophic hormone (ACTH) will significantly increase the level of free corticosterone in peripheral plasma; (2) that various stressors will elevate plasma corticosterone and (3) that hypophysectomy will abolish this latter response to stress.

*Effect of ACTH on plasma corticosterone.* To test the effect of ACTH on free corticosterone in peripheral plasma, 6-week-old male Small White turkeys were injected intramuscularly (I.M.) with 8 I.U. ACTH (Upjohn, Depo ACTH) per bird per day for 3 days. One hour after final injection, heparinized blood was drawn from the wing vein and the plasma separated by centrifugation. The results are presented in Table I. The ACTH treatment elevated free corticosterone from 12.5 μg% in the controls to 28 μg% in the ACTH treated birds. Forty-eight hours after final injection the concentration had returned to 14.2 μg% which is at or near normal levels. Thus it was firmly established that exogenous ACTH will significantly incerase the level of free corticosterone in peripheral plasma.

*Effect of stressors on intact and hypophysectomized turkeys.* Hypophysectomies were performed on the birds used in these experiments at 6 weeks of age. One week after surgery the birds were subjected to the stressors of water deprivation or cold. At termination of each experiment the birds were sacrificed, their heads removed and assigned a number. The heads were then examined to determine completeness of surgery and thus assign each turkey to one of the following groups: (1) sham operated (all of the anterior pituitary remaining; (2) ap (anterior pituitary completely removed) and (3) ap + pp (complete hypophysectomy, *i.e.*, both anterior and posterior pituitary removed). Thus, there were 4 experimental groups; controls, sham operated, ap and ap + pp on each experiment.

The results obtained with water deprivation are presented in Table II. Water was withheld from all groups for 96 hours. At the end of this period the surviving birds were again allowed free access to water.

TABLE I. Plasma Corticosterone Concentration in 6-Week-Old Small White Turkeys Injected with ACTH (10 Birds per Treatment).

| | Controls, 0.1 ml corn oil I.M./day (3 days) | 8 U ACTH I.M./bird/day (3 days) |
|---|---|---|
| | μg % | |
| 1st sample, 1 hr after last inj. | 12.5 | 28.7 |
| 2nd sample, 48 hr after last inj. | 11.9 | 14.2 |

I.M. = intramuscular. LSD (<.01) – 10.2.

TABLE II. Plasma Corticosterone Concentration and Hematocrits in 7-Week-Old Small White Tom Turkeys Subjected to Water Deprivation. (Operation performed at 6 wk of age.)

| Treatments | | 0 hr | 48 hr | 96 hr* | 168 hr (3 days after water was ret.) |
|---|---|---|---|---|---|
| | | μg % | | | |
| Controls | Corticosterone | 11.3 | 26.1 | 30.1 | 15.1 |
| | Hematocrit | 33.0 | 46.1 | 49.2 | 34.6 |
| | | (11.3) | (18.8) | (20.2) | (14.4) |
| Sham-operated | Corticosterone | 11.7 | 19.5 | 25.8 | 14.2 |
| | Hematocrit | 33.2 | 45.4 | 48.0 | 33.9 |
| | | (11.6) | (16.4) | (17.7) | (13.8) |
| ap | Corticosterone | 11.2 | 11.1 | | |
| | Hematocrit | 33.9 | 42.4 | | |
| | | (10.9) | ( 9.8) | | |
| ap + pp | Corticosterone | 12.4 | 16.3 | | |
| | Hematocrit | 35.2 | 52.0 | | |
| | | (11.6) | (10.3) | | |

( ) μg % corticosterone adjusted to a normal hematocrit – LSD (<.01%) = 5.6.
* Birds were again allowed free access to water.

There was no significant difference among the 4 groups at 0 time. The control corticosterone level increased significantly ($P < .01$) from 11.3 μg% at 0 time to 26.1 μg% at 48 hours and to 30.0 μg% at 96 hours. At 168 hours, 3 days after the surviving birds had been allowed free access to water, the corticosterone level in the control birds had returned to near normal levels. The sham operated birds also responded to the stress of water deprivation, but to a lesser extent than the intact controls. This may have been due to disturbance of the blood supply to and from the pituitary. There was a small increase in plasma corticosterone when both anterior and posterior pituitaries were removed, but no change when only the anterior pituitary was removed. It should be noted that hematocrits increased markedly in all groups at 48 hours of water deprivation, particularly in the ap + pp birds. When all corticosterone values were adjusted back to the levels that would have been attained if hematocrits had remained normal (*i.e.*, approx. 33%) (Table II) then both controls and sham operated birds showed significantly higher levels than did the ap or the ap + pp birds. Observation of these adjusted values indicates that hypophysectomy not only prevented elevation of plasma corticosterone, but also actually tended to reduce its levels from 11.3 μg% in controls to 9.8 μg% in the ap birds and to 10.3 μg% in the ap + pp birds.

All the ap + pp birds died almost immediately after the blood samples were drawn at 48 hours. The ap birds lived longer, but all died before the 96 hour collection. Observations indicated that the ap + pp birds passed tremendous quantities of fluid through their kidneys resulting in excessive water loss as urine. It is postulated that this would result in greater hemoconcentration, less efficient circulation and, therefore, less resistance to the stress of water deprivation than in the ap birds. Although the ap birds lived longer than the ap + pp birds, their early deaths indicate that they were unable to resist stress to the same degree as the control or the sham operated birds.

The effects of cold (35°-45°) on rectal temperatures and plasma corticosterone are shown in Table III. The plasma corticosterone in sham operated birds increased from 12.6 μg% at 0 time to 17.9 μg% at 24 hours. This response was prevented by hypophysectomy. As with water deprivation, the hypophysectomized birds subjected to cold stress tended to show lower than normal corticosterone levels 72 hours after the offending stressor was removed.

Although cold applied for 24 hours did not result in the death of the hypophysectomized birds, there was a significant ($P < .05$) drop

TABLE III. Effect of Cold (35°-45°F) on Plasma Corticosterone in 7-Week-Old Hypophysectomized Small White Turkeys. (Operation performed at 6 wk of age.)

| Time (hr) | Sham-operated | ap | ap + pp |
|---|---|---|---|
| | | µg % | |
| 0 | 12.6<br>(41.1) | 11.9<br>(40.6) | 12.8<br>(39.9) |
| 8 | 13.2<br>(41.0) | 11.6<br>(40.1) | 13.4<br>(38.9) |
| 24 | 17.9<br>(40.8) | 12.2<br>(39.8) | 12.5<br>(36.0) |
| 72 hr after returning to a 70°F room | 12.6<br>(41.2) | 9.9<br>(40.4) | 10.3<br>(39.5) |

( ) Rectal temperature in °C – LSD ($P < .01$) = 0.8°C.

LSD ($P < .05$) for corticosterone concentration = 5.4.

in rectal temperature of both ap and ap + pp birds. In another experiment the birds were placed in the cold and left until all the hypophysectomized birds died. Rectal temperatures were recorded every 24 hours, and the data are shown in Table IV. It is interesting to note that rectal temperatures of ap birds tended to be lower than normal and rectal temperature of the ap + pp birds was significantly ($P < .01$) lower than for the sham operated birds at 0 time. Rectal temperatures of the ap group dropped to 26.2°C at 96 hours and all birds in the group were dead at 120 hours. Rectal temperatures of the ap + pp birds dropped precipitously to 24.2°C at 72 hours and all birds in the groups were dead at 96 hours.

These data amply demonstrate that the normal turkey responds to the applied stressors of water deprivation and cold with an accelerated release and, consequently, an elevated plasma concentration of corticosterone. It has been also demonstrated that hypophysectomy prevents this response.

TABLE IV. Effect of Cold (35°-45°F) on Rectal Temperatures in 8-Week-Old Hypophysectomized Turkeys. (Operation performed at 6 wk of age.)

| Time (hr) | Sham-operated | ap | ap + pp |
|---|---|---|---|
| 0 | 41.2 | 40.8 | 39.8 |
| 24 | 41.1 | 39.4 | 37.2 |
| 48 | 41.0 | 36.7 | 33.3 |
| 72 | 41.2 | 32.0 | 24.2 |
| 96 | 41.1 | 26.7 | all dead |
| 120 | 41.2 | all dead | |

LSD ($P < .01$) = 0.9°C.

The effect of various replacement therapies on ability of hypophysectomized birds to resist stress conditions remains to be studied. The author recognizes that the thyroid may play an important role in resistance of hypophysectomized birds to stress. An attempt will also be made to study the influence of antidiuretic hormone on resistance of ap + pp birds to stress conditions. Thus the type of replacement therapies used will not only include ACTH and various adrenocorticosteroids, but also thyroxin and antidiuretic hormone preparations.

It has been postulated that the bird adrenal may be relatively autonomous and, therefore, function at a relatively high level in absence of the anterior pituitary(10). The data presented here tend to support this hypothesis in that non-stressed hypophysectomized birds exhibit plasma corticosterone levels not significantly different from normal controls. The ap birds live relatively well until subjected to severe stressors, then their inability to respond with an accelerated release of corticosterone appears to render them less resistant to the applied stressor. The relatively low level of corticosterone in ap birds which have been subjected to stress, indicates that there may be an accelerated utilization of corticosterone in response to the stressors applied.

*Summary.* Corticosterone was established as the principal free corticosteroid found in turkey plasma. A routine procedure based on the fluorescence of corticosterone was used for quantitation of free corticosterone in peripheral plasma of the turkey. Using this technic it was demonstrated that exogenous ACTH injected intramuscularly significantly increases the level of free corticosterone in peripheral plasma. Likewise, it was shown that the stressors of water deprivation and cold significantly elevate plasma corticosterone and that hypophysectomy abolishes this latter effect. It is suggested that these data indicate the validity of using plasma corticosterone concentration as a measure of severity of stress conditions in the birds. These data also support the postulate that the bird adrenal may be relatively autonomous and, therefore, functions

at a relatively high level in the absence of anterior pituitary function.

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Received April 27, 1961. P.S.E.B.M., 1961, v107.

## Demonstration of Antigen-Reagin Interaction by Free Boundary Electrophoresis Using Buffer Contaminated with Specific Antigen.[*] (26681)

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*In vitro* experiments showing the specific interaction between human reaginic serum and homologous antigen have not been demonstrated. We assume that such specific activity exists and that it can be observed through alterations in molecular behavior of reaginic serum as can be demonstrated with free boundary electrophoresis. Efforts to discover a difference between reaginic and nonreaginic serum by electrophoresis have not been clearly successful(1). Interaction of antigen with specific antibody in free boundary electrophoresis was first demonstrated by Campbell, *et al.*(2).

TABLE I. Calculation of Mobility and Percentage Composition.

| | | | | $\alpha$ | | | | $\beta$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Alb. | | $\alpha_1$ | | $\alpha_2$ | | $\beta_1$ | | $\beta_2$ | | $\gamma$ | |
| Patient | Conc. | $\times 10^{-5}$ | % | $\times 10^{-5}$ | % | $\times 10^{-5}$ | % | $\times 10^{-5}$ | % | $\times 10^{-5}$ | % | $\times 10^{-5}$ | % |
| | WSR* | | | | | | | | | | | | |
| DR | .0 | 7.08 | 58.19 | 6.01 | 3.86 | 4.45 | 8.77 | 3.56 | 5.20 | 3.19 | 13.53 | 1.25 | 10.45 |
| | .02 | 7.29 | 50.13 | 6.19 | 5.85 | 4.43 | 9.97 | 3.23 | 20.21 | — | — | 1.15 | 13.81 |
| | .04 | 6.95 | 52.48 | 5.88 | 4.95 | 4.37 | 9.05 | 3.28 | 22.46 | — | — | 1.26 | 11.02 |
| | .06 | 7.09 | 57.78 | 6.25 | 3.80 | 4.40 | 9.77 | 3.60 | 3.26 | 3.20 | 15.98 | 1.29 | 9.38 |
| | .10 | 6.82 | 59.18 | 5.73 | 4.23 | 3.87 | 8.39 | 3.41 | 5.34 | 3.04 | 14.82 | 1.07 | 8.01 |
| | WSK† | | | | | | | | | | | | |
| DR | .0 | 7.08 | 58.19 | 6.01 | 3.86 | 4.45 | 8.77 | 3.56 | 5.20 | 3.19 | 13.53 | 1.25 | 10.45 |
| | .01 | 6.77 | 54.90 | 5.72 | 4.08 | 4.20 | 10.70 | 3.22 | 6.23 | 2.90 | 13.25 | 1.05 | 10.83 |
| | .02 | 7.19 | 55.26 | 6.09 | 4.22 | 4.55 | 9.99 | 3.56 | 7.33 | 3.23 | 11.98 | 1.29 | 11.23 |
| | .04 | 6.74 | 54.79 | 5.74 | 5.92 | 4.28 | 9.11 | 3.71 | 3.58 | 3.20 | 15.32 | 1.30 | 11.30 |
| | .06 | 6.69 | 56.69 | 5.31 | 3.58 | 3.97 | 11.97 | 3.02 | 17.12 | — | — | 1.06 | 10.63 |
| | WSR* | | | | | | | | | | | | |
| UN | .0 | 6.85 | 65.80 | 4.40 | 6.34 | — | — | 3.06 | 13.32 | — | — | 1.09 | 14.52 |
| | .01 | 6.87 | 65.88 | 4.53 | 7.12 | — | — | 3.09 | 13.14 | — | — | 1.10 | 13.83 |
| | .02 | 6.82 | 64.66 | 4.46 | 8.18 | — | — | 3.09 | 13.56 | — | — | 1.23 | 13.58 |
| | .04 | 6.96 | 62.05 | 4.55 | 10.13 | — | — | 3.11 | 13.30 | — | — | 1.19 | 14.52 |
| | .06 | 6.90 | 65.27 | 4.53 | 8.06 | — | — | 3.30 | 13.50 | — | — | 1.14 | 13.18 |

$\alpha_1$ and $\beta_1$ represent total $\alpha$ and $\beta$ unless shown in respective columns.
* WSR = Water soluble ragweed.
† WSK = " " Koeleria.

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[*] This investigation was supported by a grant from Nat. Inst. Health, U.S.P.H.S.

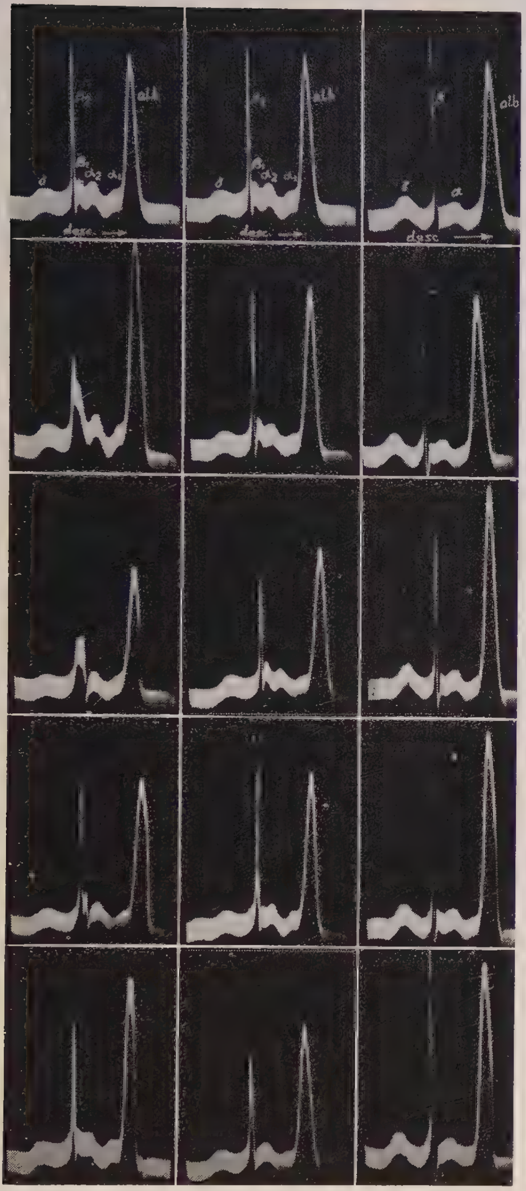

Fig. 1 Fig. 2 Fig. 3

All illustrations represent the descending boundary at end of 120 min.

FIG. 1a. Reaginic serum (DR)—buffer only.

FIG. 1b. Reaginic serum (DR); buffer contaminated with 0.02% WSR.

FIG. 1c. Reaginic serum (DR); buffer contaminated with 0.04% WSR.

FIG. 1d. Reaginic serum (DR); buffer contaminated with 0.06% WSR.

FIG. 1e. Reaginic serum (DR); buffer contaminated with 0.1% WSR.

FIG. 2a. Reaginic serum (DR)—buffer only.

FIG. 2b. Reaginic serum (DR); buffer contaminated with 0.01% nonspecific antigen (WSK).

FIG. 2c. Reaginic serum (DR); buffer contaminated with 0.02% nonspecific antigen (WSK).

FIG. 2d. Reaginic serum (DR); buffer contaminated with 0.04% nonspecific antigen (WSK).

FIG. 2e. Reaginic serum (DR); buffer contaminated with 0.06% nonspecific antigen (WSK).

FIG. 3a. Buffer only.

FIG. 3b. Nonreaginic serum (UN); buffer contaminated with 0.01% WSR.

FIG. 3c. Nonreaginic serum (UN); buffer contaminated with 0.02% WSR.

FIG. 3d. Nonreaginic serum (UN); buffer contaminated with 0.04% WSR.

FIG. 3e. Nonreaginic serum (UN); buffer contaminated with 0.06% WSR.

*Procedure.* Specific reaginic serum was migrated in a Perkin-Elmer free boundary apparatus in presence of barbital buffer contaminated with serial concentrations of Ambrosia elatior extract (WSR) within a concentration range of .01 mg-0.10 mg/ml of buffer (pH 8.6, 0.10 $\mu$). Controls consisted of (1) reaginic serum migrated in uncontaminated buffer, (2) reaginic serum migrated in buffer contaminated with an extract of a nonspecific pollen (*Koeleria cristata*) and, (3) nonreaginic serum migrated in buffer contaminated with specific antigen (Ambrosia elatior).

*Results.* Fig. 1 illustrates the visible beta globulin disturbance limited to a specific range of concentration when reaginic serum is migrated in presence of specific antigen. Fig. 2 shows the absence of this disturbance in reaginic serum in presence of a nonspecific antigen. In Fig. 3 the non-reaginic serum fails to demonstrate a change in the beta region in presence of the specific antigen. It should be noted that where specific changes in beta globulin occur (Fig. 1) similar but less conspicuous alterations may be observed in the gamma component. Calculation of the percentage composition in these findings also shows this alteration in both beta and gamma globulin (Table I).

*Discussion.* The results indicate interaction between reaginic serum and specific antigen which does not occur in the control experiments. The more impressive alteration of the beta as compared with the gamma component suggests that reaginic activity exists in this area, confirming the results of previous workers such as Cann and Loveless (3) who found that reaginic activity against insulin was contained in the beta component. The results are reenforced by observation of a gradient of activity in which an optimal zone of interaction exists between antigen excess and reagin excess. Our data has repeatedly confirmed the illustrated results, and we have also noted that the optimum concentration of the contaminating antigen varies with the serum of different allergic individuals.

*Summary.* Disturbances caused by interaction of reaginic sera of ragweed sensitive patients and its specific antigen are observed during free boundary migrations. Their respective changes of composition and mobilities of fractions are calculated and studied. Alteration of beta and, to a lesser extent, gamma globulin occur at optimum concentrations of ragweed contaminated buffer.

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Received April 28, 1961. P.S.E.B.M., 1961, v107.

# Propagation of Group A Coxsackie Viruses in Primary Human Amnion Cells. I. Cytopathic Changes Produced by 5 More Serotypes.* (26682)

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The Group A Coxsackie viruses previously reported cytopathic for tissue cultures include types 4, 6, 7, 9, 10, 11, 13, 14, 15, 16, 18, 20, 20a, 20b, 21 and 23(1,2,3,4,5,6,7). During the past 18 months we have attempted to obtain virus growth associated with cytopathic changes (CPE) in human amnionic cells with 24 serotypic Group A viruses. We were able to confirm the previous resports for all but 3 of the viruses listed above; we also obtained CPE for 5 additional serotypes heretofore unreported.

*Materials and methods. Viruses.* Twenty-four Group A serotypes and 2 subtypes (types 20a and 20b)[†] were obtained from Dr. Hildegard Plager, Division of Laboratories, New York State Dept. of Health, Albany. Virus stocks used for primary inoculation of tissue cultures were derived from suckling mice. *Antisera.* Type specific antisera were also provided by Dr. Plager. Other antisera prepared by Syverton and associates(8), and some prepared in this laboratory were available also.

*Tissue cultures.* Primary cultures of human amnion cells were prepared(9). The medium used for growth of cells consisted of medium 199 containing 20% human serum; for maintenance, washed cells were overlaid with medium 199 containing 0.5% lactalbumin enzyme hydrolysate, pH 7.3.

*Measurements.* Each mouse-adapted stock virus was inoculated into 4 cultures (0.1 ml per culture). If CPE was not noted by the 7th ± 1 day the fluid overlay was harvested and inoculated into another set of cultures. Four passages, at least were made with each serotype. The appearance of CPE was followed through additional passages until maximal CPE was obtained. At this level of passage fluid overlays were harvested and pooled for use in titration and specificity studies.

Virus titrations were done in human amnion cell cultures; successive 10-fold dilutions of virus were tested using 3 or 4 cultures per dilution. Type specificity was ascertained from serum dilution endpoints (SDE's) obtained with known serotypic antisera using approximately 100 tissue culture

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[*] Aided in part by a grant from the National Foundation and in part by funds from a contract with Nat. Cancer Inst., Department of H.E.W., P.H.S., N.I.H.

[†] Type 20, 20a and 20b are provisionally classified as type 20 viruses. Reference to 26 viruses in the text includes 20a and 20b among 24 serotypes.

TABLE I. Propagation of 5 Group A Coxsackie Viruses.

| Group A type | No. serial passages | Extent of CPE at indicated level of passage | | | | | | CPE (days) | | Titer $TCD_{50}$ per .1 ml | Serotypic neutralization | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | $\geq 6$* | First noted | Maximal | | SDE | $TCD_{50}$ |
| 3 | 12 | 1+ | 1+ | 1+ | 1+ | 2+ | 4+ | 3 | 5 | 4.7 | 256 | 250 |
| 8 | 7 | 0 | 1+ | 4+ | 4+ | 4+ | 4+ | " | 6 | 3.8 | 1024 | 80 |
| 12 | 6 | 1+ | 4+ | 3+ | 3+ | 4+ | 4+ | " | 5 | 4.7 | 500 | 370 |
| 17 | 8 | 3+ | 0 | 0 | 4+ | 4+ | 4+ | " | " | 3.5 | 630 | 20 |
| 24 | 9 | 4+ | 1+ | 1+ | 0 | 2+ | 4+ | " | 6 | 4.5 | 64 | 270 |

CPE, cytopathic effect; $TCD_{50}$, 50% tissue culture infective dose; SDE, serum dilution endpoint.

Extent of CPE: 1+, focal signs of CPE involving <50%; 2+, 50%; 3+, 75%; and 4+, 100% of cell monolayer.

* Type of change observed at passage level entered in column 2. Data for onset and progression of CPE refer to virus stocks showing maximal CPE.

infective doses ($TCD_{50}$) of virus.

*Results. Virus producing CPE.* Eighteen of the 26 Group A Coxsackie viruses produced CPE initially, or after several passages in primary cultures of human amnion cells. Fourteen strains produced unequivocal CPE during first passage; 4 viruses (types 3, 8, 12 and 16) either failed initially to cause CPE, or the changes were indecisive. Eight serotypes (types 1, 2, 4, 5, 6, 7, 19 and 22) have thus far in the study failed visibly to affect human amnion cells.

The 5 serotypes, heretofore unreported as propagating in tissue cultures, but which produced CPE in the present study were types 3, 8, 12, 17 and 24. Data obtained during studies with these viruses appear in Table I. *Kind of CPE.* The time of first appearance of CPE, and the time required for maximal CPE differed for these viruses. After onset of CPE complete lysis of cells occurred within 24 hours for some (*e.g.*, type 14), whereas others required 2 or 3 days. The pattern of CPE was quite often related to the rapidity of cell destruction. For the viruses associated with rapid CPE cells were lysed completely. But some viruses, including those listed in Table I were associated with a slower CPE. The first changes were sequestration of cells from the intact monolayer; the separated cells became shrivelled (pyknotic) and granular in appearance. While many cells remained attached to the glass surface of the roller tube, many also sloughed off into the fluid overlay.

*Titers.* $TCD_{50}$ endpoints ranged from $10^{3.5}$ to $10^{4.7}$ per 0.1 ml for the 5 viruses entered in Table I. A titer of $10^{6.5}$ was obtained for type 9, the highest yet obtained. *Serotypic specificity.* The Group A Coxsackie viruses in the fluid overlays obtained from amnion cultures were neutralized by type specific antisera. The SDE's obtained for the 5 serotypes most recently propagated in tissue cultures are given also in Table I.

*Discussion.* Nineteen of the 24 serotypes currently listed in the Group A Coxsackie viruses have been propagated in tissue cultures. We have added 5 (types 3, 8, 12, 17 and 24) viruses to the 14 previously reported to propagate *in vitro*. The viruses not yet associated with CPE include types 1, 2, 5, 19 and 22. Undoubtedly these last 5 will yield CPE when proper environmental requirements are fulfilled.

We have also studied the 24 serotypes in other cultural systems (human skin epithelium, kidney, HeLa and KB cells; monkey kidney and testis; mouse embryonic cells). Although many human cell lines supported growth of these Group A viruses, the response was not all or none. Thus, types 3 and 12 produced CPE only in human amnion, whereas types 11, 13, 14, 15, 18, 20, 20a, 20b and 21 produced CPE in each human cell line entered in the study. Other viruses, at corresponding levels of passage had cytopathic affinities between these extremes. The type 7 virus produced CPE in renal cells from monkeys and in HeLa cells, but at the 4th level of passage CPE has not been observed in amnion cells. None of the Group A viruses was cytopathic for mouse embryonic cells.

We have not been able to obtain CPE with the Albany strains, types 4 and 6. Slater and Syverton(1) obtained propagation with a type 4 (Minnesota strain) virus for 24 consecutive passages in minced mouse embryonic cells. We have not obtained either CPE, or evidence of virus multiplication on repassage of tissue culture fluids in suckling mice with the Albany type 4 virus. Lehmann-Grube and Syverton(6) also reported that type 6 propagated in human amnion cells. We failed to obtain signs of virus growth in human amnionic cells, although at the 4th passage fluid overlays obtained from cultures inoculated with this serotype produced typical disease in mice. When cultural conditions are properly fulfilled types not yet associated with CPE will probably yield. Since the media used for growth and maintenance of cells in various laboratories often differ, measurements on susceptibility and resistance of cultured cells for Group A Coxsackie viruses in different laboratories will not always be the same.

In previous reports(6,7) several Group A viruses after serial propagation in human amnion cells were, in comparison with infected mouse tissues from which they were derived, associated with a significant reduction in virulence for mice. Our results support these earlier findings. Indeed, undiluted tissue culture fluids have been required to produce illness in a fraction of infected mice. These observations point up the unreliability of using suckling mice as indicator of virus multiplication in tissue cultures. Viral antigens present in infected cells may however be delineated by the fluorescent antibody staining method(11).

*Summary.* Seventeen serotypic Group A Coxsackie viruses, representing prototypes established by Dalldorf and Sickles(5,10) have been propagated in tissue cultures. Sixteen strains produced CPE in primary human amnion cells; another (type 7) which has failed thus far to affect amnion cells produced CPE in renal cells obtained from monkeys. Among these 17 Group A viruses were 5 serotypes heretofore not associated with CPE in tissue cultures.

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Received May 1, 1961. P.S.E.B.M., 1961, v107.

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# Electron Microscopy of the Arthus Reaction, Using Ferritin as Antigen.

(26683)

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Although it is known that the Arthus phenomenon is dependent upon presence of circulating precipitating antibody(1,2,3) and that antigen is localized in blood vessel walls (4), the antigen-antibody complex has heretofore not been demonstrated in the reactive site.

The use of the electron-dense protein, ferritin, as antigen(5,6) has enabled direct visualization of the antigen-antibody precipitate in the active Arthus lesion.

*Materials and methods.* 1. Method of immunization. Horse spleen ferritin, Nutritional Biochemicals, Cleveland, Ohio, prepared by the method of Grannick(7) was used for production of Arthus sensitivity in 4-6-month-old Hartley Strain male guinea pigs and 4-6-month-old albino male rabbits.

Ten cc of a 0.1% ferritin in normal saline solution was emulsified with 10 cc of Complete Adjuvant (Freund), Difco Labs, Detroit, Mich. Rabbits were initially injected with 0.5 cc of emulsion in each of 4 subcutaneous sites. Fourteen days later they received subcutaneous injections of 2 cc of an emulsion of incomplete Adjuvant (Freund), Difco Labs, and ferritin.

Guinea pigs were immunized by intradermal injection of 1 cc of the Complete Adjuvant-Ferritin emulsion in divided doses into 8 sites in the nuchal region. Three weeks later similar injections were given using 1 cc of Incomplete Adjuvant-Ferritin emulsion.

2. Determination of specificity of immune globulin for ferritin. The animals were bled prior to, and at varying times during the sensitization, and when the lesions were biopsied. Immune and non-immune globulin was obtained by ammonium sulphate fractionation.

Immunoelectrophoresis(8) was performed to test the immunological specificity of the ferritin-antiferritin reaction. Precipitation arcs of similar contour and mobility correspinding to serum alpha globulins developed between ferritin and immune rabbit globulin and between ferritin and immune guinea pig globulin. No precipitation line formed between normal rabbit globulin and ferritin.

3. Electron microscopy of *in vitro* ferritin-antibody precipitates. After 24 hours, the 2 precipitin lines obtained by immunoelectrophoresis were cut from the agar gel and fixed, dehydrated and embedded in methacrylate for electron microscopy.

Precipitates of ferritin and immune globulin obtained in the equivalence zone were centrifuged into a pellet and prepared for electron microscopy.

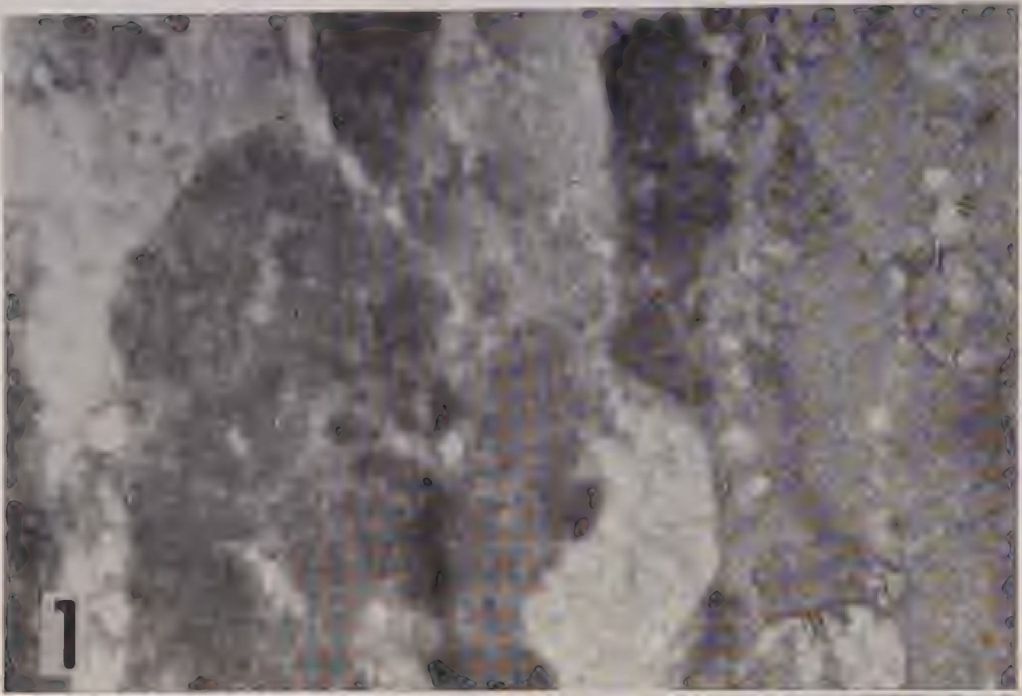
In addition precipitates of ferritin and immune globulin were obtained on grids by drying a dilute solution of ferritin on a grid and then superimposing one drop of immune globulin for 1-3 minutes.

4. Production of Arthus reactions. Arthus reactions were produced in skin and skeletal muscle of sensitized animals by intradermal or intramuscular injection of 0.1 cc-0.2 cc ferritin solution. Unsensitized animals were similarly injected with ferritin.

5. Electron-Microscopy of Arthus lesions. Active Arthus lesions induced in skin and muscle were biopsied 15 minutes, 60 minutes, 3½ hours, 12 hours, and 24 hours after injection of ferritin. Control biopsies were performed at the same intervals on skin and muscle of non-sensitized animals injected with 0.1 cc-0.2 cc ferritin solution. Half of each specimen was fixed in 10% buffered formalin for light microscopy. Blocks from the remaining tissue were fixed immediately for electron microscopy in Palade's osmium tetroxide buffered fixative(9). The skin was fixed for 2 hours and the muscle for 3½ hours. The tissue was then dehydrated in a graded series of alcohols, and embedded in 1:5 methyl-butyl methacrylate. Sections were cut on a Porter-Blum ultramicrotome, and then mounted on formvar-coated grids. The sections were examined with an RCA EMU 3B electron microscope.

*Results.* 1. Light microscopy of Arthus lesions. Typical Arthus reactions developed in the skin and skeletal muscle of sensitized rabbits and guinea pigs when challenged with ferritin. The inflammatory response progressed rapidly reaching greatest intensity within 6-8 hours. Control animals showed neither gross nor microscopic evidence of a reaction.

The vascular inflammation, thrombosis of small blood vessels, inflammatory exudate and tissue necrosis were typical of the histologic changes of active Arthus lesions(10). Small numbers of acute inflammatory cells were seen in the walls of small blood vessels in 15 minutes. The ferritin-induced reactions could not be differentiated grossly or histologically from those obtained in animals sensitized to ovalbumin. The histologic reactions in Arthus lesions produced in skeletal muscle were similar to those observed in skin.



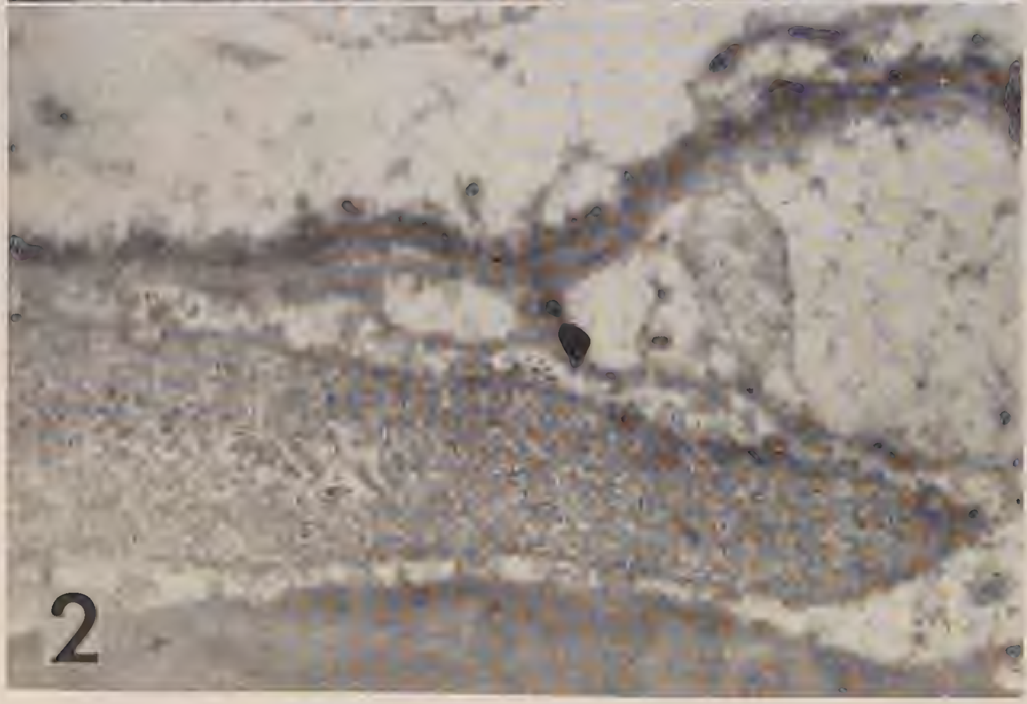


FIG. 1. Electron micrograph of Arthus reaction in guinea pig skeletal muscle. Precipitated ferritin molecules in interstitial tissue adjacent to striated muscle—3½ hr after challenge with ferritin. Magnification × 18,225.

FIG. 2. Electron micrograph of Arthus reaction in guinea pig skeletal muscle. Aggregated ferritin molecules are lined up alongside and in the basement membrane of a small blood vessel —3½ hr after challenge with ferritin. Magnification × 36,675.

2. Electron microscopic appearance of ferritin-antibody precipitates. Ferritin dried from a dilute solution on a formvar-coated grid appeared as randomly distributed discrete granules. The electron microscopic appearance of the ferritin-antibody precipitates were similar to those reported by Easty (11). The electron opaque granules were clumped and enveloped by amorphous material of lesser density, presumed to be antibody.

In control preparations of ferritin and nonimmune globulin, enveloping material was rarely seen.

3. Electron microscopic appearance of Arthus lesions. Aggregated ferritin molecules associated with an amorphous material of lesser density, comparable to *in vitro* ferritin-antibody complexes were constantly seen in Arthus lesions (Fig. 1).

The complexes were not more concentrated in the vicinity of small blood vessels than elsewhere. Nevertheless they had a distinctive circumferential distribution about the basement membranes of the vessels (Fig. 2). In some vessels ferritin aggregates were seen on either side of basement membranes and in the underlying endothelial cells.

The ferritin-antibody complex was seen in the vessels and adjacent structures of Arthus lesions biopsied as early as 15 minutes and up to 24 hours subsequent to challenge with antigen. Hemorrhage and necrosis were apparent at 3½ hours. The electron microscopic evidence of progressive tissue necrosis paralleled that seen in light microscope sections. Large aggregates of ferritin were seen within and between necrotic muscle fibers, and in the cytoplasm of cells, and mixed with erythrocytes and necrotic debris.

In control specimens there was no tissue damage. Ferritin molecules were difficult to locate in these even when biopsied 15 minutes after injection of antigen. When ferritin was seen it was scattered randomly throughout the sections and showed no localization about basement membranes of small vessels. Aggregated ferritin molecules surrounded by material of lesser electron density were not seen in control sections.

*Discussion.* The altered electron microscopic appearance of ferritin in Arthus lesions indicates that the antigen was precipitated by a protein material. The ferritin-protein complex closely resembled the *in vitro* ferritin-antibody precipitates. In view of the fact that precipitins were present in the circulating blood of the animals, it may be inferred that the ferritin-protein masses were in fact antigen-antibody complexes.

Other investigators(12) employing immunofluorescent technics have demonstrated that antigen is localized in the subendothelial region of blood vessels in the Arthus reaction. The findings in the present study differ somewhat in that the antigen-antibody complexes were found not only in the vessels but also in adjacent tissues.

The circumferential arrangement of ferritin-antibody complexes in and about the basement membranes of small blood vessels suggests that this site of localization is a prelude to the vascular necrosis which is such a prominent histological feature of the reaction.

The prominence of ferritin-antibody complexes in interstitial tissue, as compared to the minimal interstitial fluorescence of antigen in the immunofluorescent technics, can be accounted for by differences in the methods employed.

In the former instance all the antigen and antibody which react to cause the Arthus lesion is visible, whereas in the latter only the unbound antigen or antibody is available for fluorescence.

*Summary.* Arthus lesions were produced in skin and striated muscle of guinea pigs and rabbits sensitized to ferritin. Biopsies of these lesions were then examined with the electron microscope. Electron micrographs of lesions obtained as early as 15 minutes following challenge with antigen demonstrated ferritin surrounded by amorphous material of lesser electron-density comparable to *in vitro* ferritin-antibody precipitates. Ferritin-antibody precipitates were widely distributed throughout the lesions and were preferentially localized in and on either side of the basement membranes of small blood vessels.

Immunoelectrophoresis was kindly performed by Dr. Alan Solomon, Metabolism Service, General

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Received May 2, 1961. P.S.E.B.M., 1961, v107.

# Tetracycline Accelerates Elimination of Strontium from Bones in Mice.*

## (26684)

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The antibiotic tetracycline, identified in tissues by its golden fluorescence, is preferentially deposited in growing bone(1), and has been shown to inhibit skeletal formation in the chick embryo(2). It has been hypothesized that the tetracyclines interfere with cellular and enzymatic processes by virtue of their affinity for inorganic cations, especially calcium and magnesium(3). We have investigated the effects of tetracycline on strontium metabolism in adult mice, using Sr-85, a gamma emitter, as tracer. Aside from its importance in radioactive fallout (as the isotope Sr-90), strontium has gained increasing attention as a tracer for studying bone metabolism, being handled in the body qualitatively similarly to calcium(4).

In this experiment, 19 Swiss Webster mice were given single injections of $Sr^{85}(NO_3)_2$, 1 $\mu$c each, specific activity 200 $\mu$c/mg. The control group, consisting of 10 animals, received no other treatment. The 9 mice in the second group were given daily injections of tetracycline, 150 mg/kg, starting on the 4th day prior to Sr-85 injection, and continuing to the 25th day after Sr-85 injection, when all the animals were sacrificed. The amount of Sr-85 in each mouse each day was measured with an external gamma detector (NaI scintillation counter). Average values of total body Sr-85 against time are shown in Fig. 1. Each curve has a fast component, representing direct excretion from the body of a portion of the initial dose, and a slower component, representing gradual elimination of the portion incorporated into bone. Extrapolation of the slow exponential yields the fraction of the original dose which was deposited in bone. At autopsy, no significant

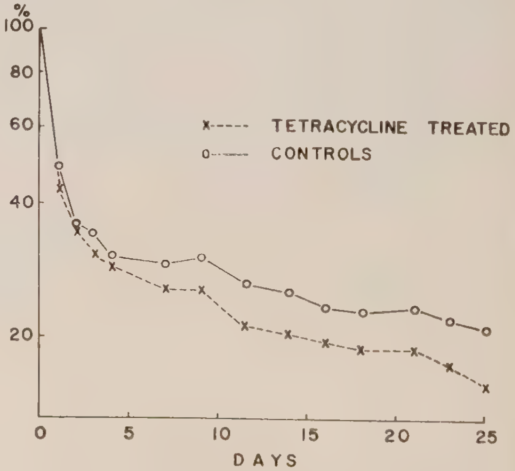

FIG. 1. Turnover of $Sr^{85}$ in tetracycline treated mice.

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* Supported by Am. Cancer Soc. Grant T134 and Damon Dunyon Fund.

TABLE I.

| | Control | Tetracycline | P |
|---|---|---|---|
| Half-time in days of fast component | .65 ± .17 | .61 ± .12 | .58 |
| % of dose incorporated in bone | 35 ± 7 | 33 ± 4 | .55 |
| Half-time in days of slow component (elimination of Sr-85 from bone) | 35 ± 4 | 22 ± 2 | .01 |
| % of dose in whole mouse on 25th day | 21 ± 4 | 16 ± 2 | .01 |
| % of dose in femurs on 25th day | 1.65 ± .43 | 1.16 ± .06 | .01 |

Sr-85 was present in any tissue except bone. The Sr-85 content of femurs from each mouse was determined. Comparison of the two group averages (± S.D.) is set forth below. P is the probability of the difference between groups having occurred by chance.

It appears then that under the conditions of this experiment parenterally administered tetracycline accelerates elimination of strontium from bones, while not significantly affecting the amount deposited in bones. Dosage and time dependence of this effect is being further studied.

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Received May 4, 1961. P.S.E.B.M., 1961, v107.

# Glucose Uptake by Epididymal Fat Pads from Rats Fed Glucose, Lactose or a Glucose-Galactose Mixture. (26685)

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The carcass fat content of growing and mature rats fed glucose is about 40% greater than that of those fed lactose. This difference persists even if the weight of the glucose-fed animals is kept equal to that of the lactose-fed group by food restriction(1). Fat deposition in rats fed prehydrolyzed lactose, *i.e.,* a 50-50 mixture of glucose and galactose, is also higher than in lactose-fed rats(1).

Recent research(2) indicates that conversion of blood glucose to lipid by adipose tissue is of importance to an understanding of the biochemical basis for obesity. Interest in the mechanism responsible for differences in fat deposition led us to compare rate of glucose uptake by the adipose tissue from rats fed various carbohydrates.

*Materials and methods.* Male Sprague Dawley rats were fed a diet containing glucose, lactose or a 50-50 mixture of glucose and galactose, 52; casein, 24; fat, a mixture of vegetable oils and oleo oil, 20; salt mixture, Hubbel, Mendel, and Wakeman, 4; plus an adequate vitamin mixture(1).

The epididymal fat pads from rats, killed by a blow on the head, were placed as rapidly as possible in 5 ml of Krebs-Ringer bicarbonate solution at 37° with a glucose concentration of 5 $\mu$M per ml, contained in a 50 ml Erlenmeyer flask. When added, insulin concentration was 0.1 unit per ml. Weight of the tissue was estimated by weighing the flask plus contents to the nearest 10 mg before and after addition of the tissue. After exposing to a water saturated mixture of 95% $O_2$-5% $CO_2$ at 37° for 5 minutes the flasks were kept at 37° with gentle agitation at 60 cycles per minute for 3 hours. Glucose was determined by Nelson's method(3) after deproteinization with $ZnSO_4$ and Ba $(OH)_2$; nitrogen was determined by the Kjeldahl method after defatting the tissue with ace-

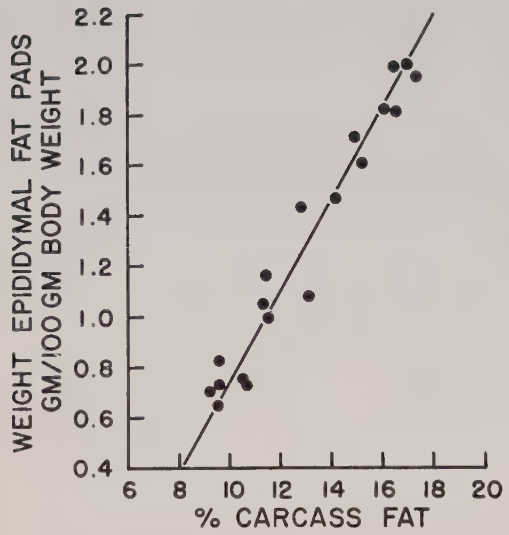

FIG. 1. Correlation between carcass fat content and weight of epididymal fat pads, % carcass fat = 6.12 + 5.49 • weight of epididymal fat pads. Average error of estimate of carcass fat content is 0.54%, range 0-1.1%. Each point represents avg of an experimental group of 5-10 weanling rats fed diets containing 52% of various carbohydrates for 6 wk.

tone and ethanol. All activity determinations were done in duplicate.

Total carcass fat was estimated from the weights of the epididymal fat bodies using the data of Fig. 1 obtained in previous studies(1). These data show that the close relationship between weight of the epididymal fat pads and carcass fat content reported by others(4,5) also applies when various carbohydrates are fed.

*Results and discussion.* The data obtained are shown in Table I. As is customary, calculations of activity of the tissues are based on nitrogen content, a measure of the number of cells. In the absence of insulin, rate of glucose uptake by the epididymal fat bodies from rats fed lactose was lower, though not significantly so, than in the glucose fed group. In the presence of insulin, glucose uptake of this tissue from rats fed lactose was significantly less than that of those fed glucose or a 50-50 mixture of glucose and galactose.

Reduction in food intake sufficient to restrict the weight gain of a glucose-fed group to that of a group fed lactose did not result in a reduction of rate of glucose uptake, indicating that the higher rate of glucose uptake by adipose tissue of rats fed glucose is not related to differences in food intake be-

TABLE I. Relationship between Dietary Carbohydrate and Metabolic Activity of Adipose Tissue.

| | Exp. I | | Exp. II | | | |
|---|---|---|---|---|---|---|
| Diet | Lactose* | Glucose* | Lactose* | Glucose* | Glucose & galactose* | Glucose, reduced intake† |
| | a | b | c | d | e | f |
| Initial body wt | 132 | 132 | 65 | 65 | 65 | 65 |
| Final " " | 319 | 352 | 290 | 331 | 306 | 280 |
| Epididymal fat bodies, g/100 g | 1.10 | 1.88 | .92 | 1.80 | 1.40 | 1.66 |
| Epididymal fat bodies, mg N/g tissue | 2.0 | 1.5 | 2.0 | 1.6 | 1.8 | 1.9 |
| % carcass fat§ | 12.2 | 16.5 | 11.2 | 16.0 | 13.8 | 15.2 |
| Glucose uptake, $\mu$M/mg N‡ | | | | | | |
| Without insulin | .67 ±.2 | .81 ±.14 | | | | |
| With " | 2.17 ±.27 | 4.13 ±.47 | 3.79 ±.17 | 5.88 ±.30 | 4.96 ±.42 | 6.46 ±.46 |

P values

| Without insulin | With insulin | | | | |
|---|---|---|---|---|---|
| a *vs* b = .6 | a *vs* b = <.01 | c *vs* d & f = <.001 | c *vs* e = <.05 | d *vs* e = .1 | d *vs* f = .3 |

7 rats/group, 6 in Group c.

* *Ad libitum* feeding.

† Food intake reduced sufficiently to maintain weight close to that of lactose-fed group.

‡ Avg ± S.E. of mean.

§ Estimated from data of Fig. 1.

tween the dietary groups. The finding that the feeding of a 50-50 mixture of glucose and galactose resulted in a higher rate of glucose uptake indicates that the effect of lactose involves metabolic effects other than those of the absorbed monosaccharides.

Previous studies have shown that the *in* vitro rate of glucose uptake by liver tissue (6) and liver enzyme activities(7) varies after feeding different carbohydrates. The present results show that the glucose metabolizing activity of adipose tissue was also influenced by the carbohydrate of the diet.

The differences in glucose uptake in the *ad libitum* fed groups were directly related to weights of the epididymal fat bodies or estimated total fat content of the carcass. This relationship is in agreement with the finding that rate of triglyceride synthesis is directly related to rate of glucose metabolism by adipose tissue(2).

*Summary*. Comparisons were made of the *in vitro* rate of glucose uptake by the epididymal fat pads from rats fed lactose, glucose or a 50-50 mixture of glucose and galactose. Glucose uptake of insulin-stimulated adipose tissue from rats fed lactose was significantly lower than in the other dietary groups. A direct relationship was observed between rate of glucose uptake and weight of the epididymal fat pads or estimated carcass fat content.

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Received May 8, 1961. P.S.E.B.M., 1961, v107.

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# Transfer of Tolerance Induced by Parabiosis to Isologous Newborn Mice.* (26686)

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Tolerance of skin homografts has been induced in adult inbred mice by establishing celomic parabiosis between members of various homologous strains. For example, adult mice of the Z§ strain joined to homologous (A × Z) $F_1$ hybrids or to mice of the Ce strain resulted in tolerance whereby the Z partner accepted skin homografts taken from either (A × Z) $F_1$ hybrid cross or Ce strain donors(1). Further experimentation demonstrated that the length of time during which parabiosis had to be maintained to achieve tolerance varied according to the strains of mice joined(2). Induction of tolerance in adult mice by this procedure was interpreted as being the result of a continuous exchange of transplantation antigens in the form of intact cells between members of the pair resulting in a suppression of the homograft reaction thus permitting establishment in the homologous host of replicating cells containing the corresponding transplantation antigens(3,4).

On the other hand, our previous reports have indicated that immunological tolerance of tissue homografts induced by antigenic exposure during the neonatal period can be transferred to isologous mice by injecting these animals at birth with viable spleen cell

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* Aided by grants from N.I.H., Am. Cancer Soc., U. S. Public Health Service and Minn. Div. Am. Cancer Soc.

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§ Z is used to designate mice of the C3H strain originally obtained from Dr. J. J. Bittner's mouse colony.

TABLE I. Transfer of Tolerance Induced by Parabiosis to Isologous Newborn Mice.

| Spleen donor strain* | Host strain | No. of mice accepting AZ $F_1$ homograft/No. of animals grafted | % |
|---|---|---|---|
| Z tol. of AZ $F_1$ (parabiosis 30 days)† | Z | 0/20 | ( 0) |
| *Idem* (parabiosis 150 days)‡ | " | 19/28 | (68) |
| None | " | 0/24 | ( 0) |

* The dose of spleen cells inj. intrav. into newborn Z strain mice was 4-5 million cells/mouse.
† Tolerance induced by Z—AZ $F_1$ parabiosis maintained during 30 days.
‡ Tolerance induced by Z—AZ $F_1$ parabiosis maintained during 150 days.

suspensions taken from adult tolerant donors. In other words, tolerance of "B" strain tissue induced at birth in "A" strain mice can be transferred to isologous "A" strain animals by injecting these mice at birth with spleen cells taken from "A" tolerant of "B" adult donors(5). This was interpreted as an indication that in the tolerant host all of the transplantation antigens presumably in the form of many intact cells derived from the original donor are present in the reticulo-endothelial system of the tolerant individual.

In view of these results it was considered important to ascertain whether or not tolerance of tissue homografts induced in adult mice by means of parabiosis could also be transferred to isologous animals by injecting them at birth with viable spleen cells taken from tolerant donors. It was reasoned that if tolerance induced by parabiosis could indeed be transferred in this fashion it would be a strong indication that the parabiosis-induced tolerance in adult mice as well as that obtained in neonates may be the result of persistence of homologous cells in the reticulo-endothelial system. The results reported herein seem to support this concept.

*Method.* Adult mice of the Z strain and $F_1$ hybrids resulting from the cross between A strain females and Z males were used. When mice were approximately 3 months of age a "Z—AZ $F_1$" celomic parabiosis was performed by the technic previously described(3). Parabiotic pairs were housed in plastic cages containing one pair per cage and fed laboratory chow and tap water. At various time intervals, namely 30 and 150 days following establishment of parabiosis groups of parabiotic pairs were surgically separated and 8 days later the Z strain parabiont received a full-thickness abdominal skin homograft taken from donors of the AZ $F_1$ hybrid cross. The technic of skin grafting was the same as previously described (6). Mice accepting the skin homografts for a period of time of at least 3 months were considered as tolerant and submitted to removal of their spleens to prepare a spleen cell suspension in Ringer-Locke's saline solution containing approximately 10 million viable spleen cells per 0.1 cc. This suspension was immediately injected intravenously into newborn mice of the isologous Z strain in a dose of 0.05 cc per mouse. The technic for preparation of the spleen cell suspension was described previously(6) and the method of intravenous injection in newborn mice was the same as that recommended by Billingham and Brent(7).

Injected mice were raised by their own mothers and at approximately 2 months of age received a full-thickness abdominal skin homograft taken from adult AZ $F_1$ hybrid donors. After skin grafting, mice were singly housed in plastic cages and fed laboratory chow and tap water. Grafts were inspected daily and success or failure scored at the end of the 5th month following transplantation.

*Results.* Results are listed in Table I and Fig. 1. Of the group of 20 animals injected intravenously at birth with viable spleen cells taken from Z strain donors made previously tolerant of AZ $F_1$ hybrid tissue by virtue of parabiosis of 30 days duration, none were tolerant and rejected the AZ $F_1$ hybrid skin homografts. In contradistinction, 19 of 28 mice similarly injected at birth with spleen cells derived from Z mice made tolerant of AZ $F_1$ hybrid by "Z—AZ $F_1$" parabiosis maintained for 150 days became tolerant and successfully accepted AZ $F_1$ homologous skin grafts. As expected, none of the non-injected

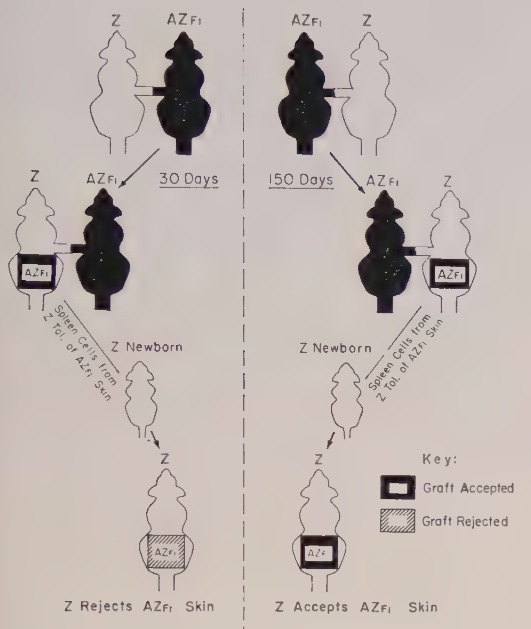

FIG. 1. Transfer of tolerance induced by parabiosis. Spleen cells taken from mice of Z ($C_3H$) strain made tolerant of AZ $F_1$ hybrid tissue by virtue of Z ($C_3H$)—AZ $F_1$ parabiosis of 150 days duration are able to induce tolerance of $F_1$ strain when inj. into newborn mice of the Z ($C_3H$) strain. However, if inj. spleen cells are taken from Z ($C_3H$) donors tolerant of AZ $F_1$ hybrid tissue as a result of parabiosis of 30 days duration, tolerance of AZ $F_1$ tissue could not be obtained.

controls accepted the homologous $F_1$ hybrid skin transplant.

*Discussion.* The results are of interest from several points of view. They confirm our previous reports that tolerance of homologous tissue transplants can be induced in adult mice by establishing a celomic parabiosis between members of certain homologous strains(1,2,3). The mechanism whereby tolerance is achieved by this method was postulated to be the result of a continuous exchange between both parabionts of all the transplantation antigens in the form of intact cells. If this exchange is maintained for a sufficient length of time permanent establishment of these cells in the homologous host occurs and lasting immunological tolerance is the rule. The results herein reported also demonstrate that tolerance of AZ $F_1$ skin homograft induced in adult Z mice by the parabiosis procedure can be transferred to isologous Z strain mice by intravenously injecting these animals at birth with spleen cells taken from the Z tolerant of AZ $F_1$ hybrid donors. It is interesting to note that tolerance can be transferred only when the spleen cells were derived from mice made tolerant by virtue of Z—AZ $F_1$ parabiosis of 150 days duration and not at all when these cells were taken from tolerant animals resulting from parabiosis of only 1 month duration. Transfer of tolerance to isologous mice by injection of spleen cells during the neonatal period can be interpreted as being the result of transfer of homologous AZ $F_1$ cells in residence in the reticulo-endothelial system of the chimeric tolerant Z donor resulting from the parabiotic exchange of cells. The fact that transfer of tolerance was successfully achieved when the donors of the spleen cells had been maintained in parabiosis for 150 days and failed when the spleen cells were derived from similar donors maintained in parabiosis for only 30 days can be interpreted as a function of the actual number of homologous cells in residence in the reticulo-endothelial system of the donor animal at the time the transfer of tolerance was attempted. In this regard it is feasible that the number of homologous cells present in the spleen of the tolerant donor after 30 days of parabiosis, although enough to maintain the state of non-reactivity, was not quite sufficient to induce tolerance after transferring these cells to isologous newborn mice. In contradistinction, the spleen derived from tolerant donors by virtue of parabiosis of 150 days duration might contain many more homologous cells, in sufficient number to induce tolerance when transferred to isologous newborn individuals.

It seems clear, therefore, that tolerance of homologous tissue grafts induced by the parabiosis procedure in adult mice of certain donor-host strain combinations results from an exchange of cells containing all the transplantation antigens establishing residence and almost certainly replicating themselves in the reticulo-endothelial system of the homologous host. The transfer of this form of tolerance by injecting isologous newborn animals with spleen cells derived from tolerant donors supports this interpretation.

*Summary*. Immunological tolerance of AZ $F_1$ hybrid tissue homograft induced in adult Z mice by virtue of parabiosis of 150 days duration can be transferred to isologous Z strain mice by injecting these animals at birth with spleen cells taken from Z donors tolerant of AZ $F_1$ tissue. Tolerance resulting from parabiosis of only 30 days duration failed to be transferred by the same procedure.

In the light of these results the mechanism of tolerance induced in adult individuals by the parabiosis procedure is discussed.

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Received May 8, 1961. P.S.E.B.M., 1961, v107.

## Effect of Hydrocortisone on Human Cells in Tissue Culture.* (26687)

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The cytotoxic and growth-inhibiting effects of adrenal cortical steroids on various cell strains growing *in vitro* have been observed frequently(1-5). Recently we reported that hydrocortisone inhibited cell growth in proportion to the concentration used(6). The purpose of the present study was to determine the quantitative effects of hydrocortisone on protein synthesis and on nuclear multiplication and to investigate the qualitative effects on enzymes of cells in tissue culture.

*Methods*. A strain of cells derived from human liver by Chang(7) was grown in stock culture in 95% medium 199 and 5% horse serum. The cells were removed from the flasks by replacing the growth medium with 0.0125% trypsin in Hanks' balanced salt solution(8) for 15 minutes at 35°C and centrifuging the resulting cell suspension for 5 minutes at 1000 rpm. The supernatant was removed, the cells were washed in medium 199, and resuspended in medium 199 with 5% horse serum in a concentration of 100,000 cells/ml. Five ml aliquots were placed in T-flasks for studies on the rates of nuclear multiplication and protein synthesis. All cultures were incubated at 37°C for 24 hours, after which the culture medium was removed from the flasks and the flasks rinsed with 5.0 ml balanced salt solution. Determinations of protein nitrogen content of each of 3 cultures were made with a phenol reagent according to the method of Oyama and Eagle(9). Crystalline bovine albumin was used as a standard. The number of nuclei was determined in 4 different samples from each of 3 flasks by the method of Sanford *et al.*(10) using 6% citric acid to remove the cytoplasm and 0.1% crystal violet to stain the nuclei.

The culture medium to be tested was added to the remaining flasks; 6 flasks were used for each group.

At the end of the experiment 3 flasks from each group were used for determination of protein content and 3 flasks for the number of nuclei.

For microscopic studies coverslips were placed into Leighton tubes containing 100,000 cells suspended in 1 ml of medium 199 with 5% horse serum. After incubation for 24 hours at 37°C, the culture medium was

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* Supported in part by grants from Am. Cancer Soc. and USPHS.

TABLE I. Effect of Concentration of Hydrocortisone on Growth of Cells.

| Hydrocortisone, µg/ml | Incubation period, hr | Nitrogen, µg/ml ± S.E. | | Nuclei, 1000/ml ± S.E. | | Nitrogen/cell, mµg |
|---|---|---|---|---|---|---|
| 0 | 0 | 11.1 | .14 | 81 | 2.1 | .138 |
| 0 | 72 | 14.2 | .34 | 219 | 11.8 | .065 |
| 50 | " | 14.3 | .50 | 168 | 5.5 | .085 |
| 100 | " | 11.7 | .42 | 114 | 7.4 | .103 |
| 200 | " | 12.8 | .59 | 91 | 3.4 | .141 |

removed and the tubes were rinsed with balanced salt solution. Culture media corresponding to those used in the flasks were added to the Leighton tubes and the culture incubated at 37°C. At the end of the experiment the coverslips were removed from the Leighton tubes. One coverslip from each group was stained with hematoxylin and eosin; some coverslips were stained by the Feulgen reaction. The remaining coverslips were used for histochemical demonstration of succinic dehydrogenase(11), diphosphopyridine nucleotide diaphorase(12), glucose-6-phosphate dehydrogenase(13), acid and alkaline phosphatase, and esterase(14).

*Results. Effect of concentration of hydrocortisone.* The experimental groups were cultured in medium 199 containing 50, 100 and 200 µg/ml hydrocortisone and controls were grown in medium 199 alone. At the end of 72 hours of incubation at 37°C the experiment was terminated. In each group protein content was determined in each of 3 flasks, and number of nuclei determined in each of the remaining 3 flasks.

In the controls nitrogen content had increased from an initial value of 11.1 µg/ml to 14.2 µg/ml (Table I). In the cultures grown in the medium with varying concentrations of hydrocortisone nitrogen values varied from 14.3 to 11.7 µg/ml. In the controls the number of nuclei had increased from 81,000 to 219,000/ml, while in the experimental cultures with hydrocortisone concentrations of 50, 100 and 200 µg/ml the nuclei had increased to 168,000, 114,000 and 91,000/ml respectively. Thus degree of inhibition of nuclear multiplication was proportional to concentration of the hormone. However, the nitrogen content of the cultures did not show an equivalent reduction. This difference in effect of hydrocortisone on the nucleus as compared with the cytoplasm was reflected in the nitrogen/cell values which had progressively increased with increasing concentrations of hydrocortisone.

*Effect of length of incubation.* In these experiments the dosage of hydrocortisone was 150 µg/ml in all experimental groups. Each group was incubated for 24, 48 or 72 hours. At the end of each period protein content and number of nuclei were determined in experimental and corresponding control groups.

Both number of nuclei and nitrogen content of the cells grown in media with hydrocortisone were less than those of the controls. Moreover, the differences between the experimental groups and their respective controls tended to increase with longer periods of incubation.

In the controls there was a progressive increase in nitrogen content and number of nuclei proportional to the length of incubation period (Table II and Fig. 1). The initial value for nitrogen content was 10.8 µg/ml and for number of nuclei, 77,000/ml. These values increased to a maximum nitrogen content of 15.2 µg/ml and to 178,000 nuclei/ml at 72 hours. In the experimental group cultured in 150 µg/ml hydrocortisone nitrogen content was 10.2 µg/ml at 24 hours, 10.8 µg/ml at 48 hours, and 12.2 µg/ml at 72 hours. Numbers of nuclei in all of the experimental groups were less than those in the corresponding controls, *i.e.*, 86,000, 119,-

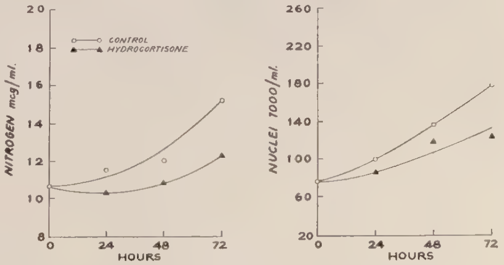

FIG. 1. Cells grown in medium 199 with 150 µg/ml hydrocortisone for 24, 48 and 72 hr.

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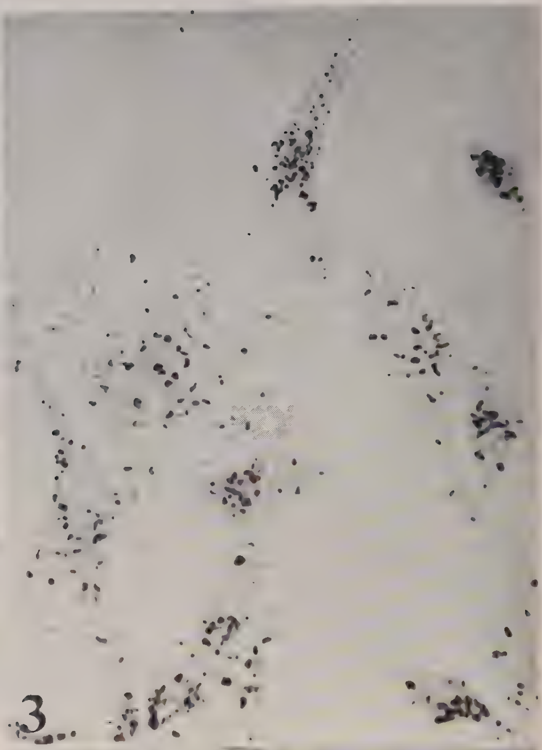

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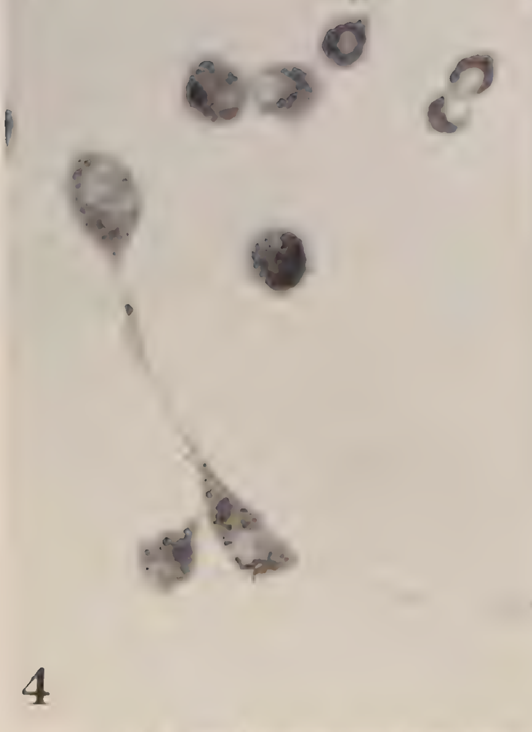

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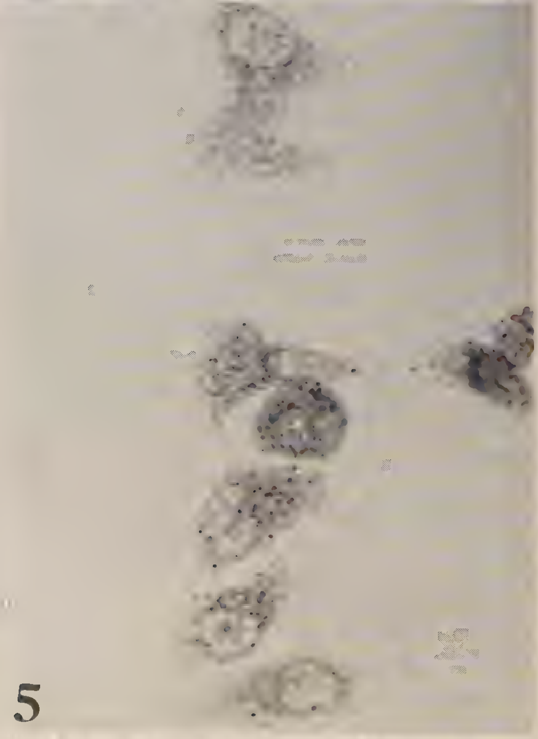

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FIG. 2. Control cells cultured in medium 199 with small granules of nonspecific esterase-positive material in the cytoplasm. × 450.

FIG. 3. Experimental cells cultured in medium 199 with 150 $\mu$g/ml hydrocortisone. Cytoplasm contains an increased number of esterase-positive granules. × 450.

FIG. 4. Control cells cultured in medium 199. Cytoplasm contains a large amount of succinic dehydrogenase-positive granules. × 450.

FIG. 5. Experimental cells cultured in medium 199 with 150 $\mu$g/ml hydrocortisone showing a decreased amount of succinic dehydrogenase-positive material. × 450.

TABLE II. Effect of Length of Incubation on Growth of Cells.

| Group | Incubation | Nitrogen, $\mu g$/ml ± S.E. | | Nuclei, 1000/ml ± S.E. | | Nitrogen/cell, m$\mu g$ |
|---|---|---|---|---|---|---|
| Controls | 0 | 10.8 | .43 | 77 | 3.3 | .140 |
| | 24 | 11.5 | .14 | 100 | 5.2 | .115 |
| | 48 | 12.0 | .00 | 137 | 5.4 | .087 |
| | 72 | 15.2 | .46 | 178 | 10.4 | .085 |
| Hydrocortisone, 150 $\mu g$/ml | 24 | 10.2 | .38 | 86 | 4.0 | .120 |
| | 48 | 10.8 | .58 | 119 | 8.7 | .090 |
| | 72 | 12.2 | .38 | 124 | 3.1 | .098 |

000 and 124,000/ml.

*Morphologic and histochemical changes.* There were consistent differences between hydrocortisone-treated cells and controls. The nuclei of the cells treated with hydrocortisone were larger, more irregular, and showed greater variation in size than the controls. These nuclei were stained less intensely by hematoxylin; multinucleated cells were more abundant. Mitoses were about two-thirds as numerous in hydrocortisone-treated cells as in controls. The cytoplasm of the hydrocortisone-treated cells was more abundant but less dense than that of the controls.

In the hydrocortisone-treated cells the nuclei stained less intensely with the Feulgen reaction. Acid phosphatase and nonspecific esterase (Fig. 2 and 3) had increased; succinic dehydrogenase (Fig. 4 and 5) and glucose-6-phosphate dehydrogenase were decreased in the cells treated with hydrocortisone. Alkaline phosphatase and diphosphopyridine nucleotide diaphorase reactions showed no difference in experimental and control groups.

*Summary.* Hydrocortisone inhibited the growth of cells in a synthetic medium. Degree of inhibition was proportional to concentration of the hormone and duration of exposure. The inhibition in nuclear multiplication was relatively greater than the inhibition of protein synthesis. The cells grown in media with hydrocortisone had larger, more variable nuclei, increased amounts of acid phosphatase and nonspecific esterase and decreased amounts of succinic dehydrogenase and glucose-6-phosphate dehydrogenase.

The authors gratefully acknowledge the technical assistance of Miss Virginia Jentoft and Miss Barbara Jennings.

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Received May 8, 1961. P.S.E.B.M., 1961, v107.

# Paper Electrophoretic Study of Protein-Losing Secretions from Explanted Gastric Mucosa in Rats.* (26688)

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Application of paper electrophoresis to analysis of the normal human gastric juice has resulted in identification of 8 to 11 large molecular components(1,2).

The most anodic of the components, P, represents pepsin, while the central electrophoretic components are mucoproteins which contain proteins or proteoses, hexoses, hexosamine, fucose, uronic and sialic acids as well as sulfates, and stain with amido black 10B, SF light green and periodic acid Schiff stains (3,4). Of the latter group, the most anodic one (glandular mucoprotein(5)) is supposedly derived from the fundic glands, while the central electrophoretic components, $M_2$, $M_3$, $M_4$ and $X_1$, represent mucous materials and their degradation products, derived from surface epithelium mucus and also, possibly, from the pyloric glands(2,6,7). The materials on the extreme cathodic side of the electrophoretic partition are large molecular peptides, probably derived from enzymic degradation of serum proteins(8).

In the search for *direct* information on cellular origin of these large molecular components of gastric juice, we used the method described by Toovey, Heller and Webster(9) and Webster, Toovey and Skoryna(10) for exteriorization of a portion of the rat glandular stomach onto the anterior abdominal wall. Histological studies(9,10) had demonstrated that after a lapse of a few weeks, the surface epithelium of the exteriorized gastric flap completely covered the surface of the explant, effectively blocking the orifices of the gastric glands. This enabled these investigators to obtain secretions from the gastric mucosa free of gastric glandular components. A preliminary abstract of our investigations has been published(11).

*Method.* The studies were carried out on adult male rats of the Royal Victoria Hospital strain, a) with ligated cardia and pylorus, and b) with gastric explants.

*Rats with cardia and pylorus ligation.* The animals were starved for 12 hours, then anesthetized with nembutal. After irrigation of the stomach with normal saline to remove food remnants, the cardia was ligated at the junction of esophagus and forestomach. The pylorus was then ligated and the laparotomy wound closed by continuous suture. Care was taken that the vagus nerve and blood vessels were not included in the ligature. After 24 hours, the animals were again laparotomized. At this time, the gastric pouch enclosed between the cardiac and the pyloric ligatures usually bulged with secretions. The esophagus and duodenum were transected beyond the ligatures, the gastric pouch removed and, through a small incision in the forestomach, the secretion was emptied into a beaker. Eleven pools, each of 14 to 22 cc, were obtained, each representing the 24-hour secretion of several rats. Only pools of perfectly clear gastric juice, free of blood, were analyzed.

*Rats with explanted gastric wall.* The animals were starved for 12 hours, then anesthetized with nembutal. A portion of the anterior and posterior gastric walls adjoining the greater curvature was separated into a flap with maintained blood supply. The continuity of the stomach was then restored by closing the transection line with double continuous suture. The flap of the glandular stomach was then exteriorized and the abdominal wall closed around the mesentery, care being taken not to impair the blood supply of the flap. The serosa of the flap was then sutured to the skin of the anterior abdominal wall.

Following explantation, progressive inflammation and destruction of the explanted mucosa was observed. After the second week,

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* Supported by Am. Cancer Soc. Research grant-in-aid and Inst. of Arthritis and Metab. Dis., N.I.H., P.H.S., grant.

a new, low cuboidal epithelium could be seen, extending from the remaining intact mucosa at the mucocutaneous junction of the infolded edges of the flap. This newly-formed surface epithelium was almost entirely covered by a heavy lining of mucus. The surface not covered by epithelium and mucus was fibrous, or exhibited chronic ulcerations due to repeated traumatization of the exposed gastric surface. As shown by microscopic study, the deep glandular elements were retained, and often proliferated into the lamina propria. The ducts of the glands were dilated, however, since their orifices were blocked by the newly-formed surface epithelium. This rapid epithelialization was probably due to the higher division rate of surface epithelial cells as compared with mucous neck cells(12). The glandular elements of the mucosa in the explants thus either underwent atrophy or their orifices were shut off by new surface epithelium. After 4 weeks, the explant usually measured 1 × 1 cm. The animals were placed in Bollman cages and the fluid secreted from the flaps was collected in a glass cup or funnel, beginning 8 weeks after explantation.

The rats were kept on a standard Purina meal diet, with water *ad libitum* except for the starvation period. Total number of rats with explanted gastric mucosa, the secretions of which could be used for the present experiment, was 12. From each rat 10 to 12 collections were obtained over a period of about a month, volume of each animal's secretion totalling from 10 to 13 cc. Each collection was immediately centrifuged and the supernatant stored in a refrigerator. After pooling, total collection from each rat was divided into 2 portions. One was frozen and the other was extensively dialyzed at 4°C for 24 hours against frequent distilled water changes, then lyophilized. The same procedures were applied to the processing of the secretions of the stomachs with ligated cardia and pylorus.

The frozen native gastric juices were used for determination of HCl, pepsin, mucoprotein and mucoproteose, while the dialyzed and lyophilized juices were used for paper-electrophoresis. Pepsin activity was determined at pH 1.5 by modification of the hemoglobin method(13)·; cathepsin, by the same method, at pH 3.5; mucoprotein and mucoproteose, by the method of Glass and Boyd(14), and paper-electrophoresis of the gastric juice by the method of Glass, Stephanson and Rich(2), using Spinco cell, borate buffer of pH 9.0 and ionic strength 0.24, Whatman No. 1 paper, 0.4 mA/cm at 120 volts, for 5½ hours. The strips were stained with amido black 10B(2), SF light green (15) and PAS(16), then traced and integrated in Analytrol, using 575 mμ filter for amido black, 620 mμ for SF light green, and 550 mμ for the PAS stain.[†]

*Results. Chemical assays of gastric secretion.* The gastric juices from the ligated rat stomachs contained acid and pepsin, as shown in Table I. Gastric juices from the explants, however, contained no free HCl, pepsin or 'cathepsin'. 'Glandular mucoprotein' was either absent, or present in traces (0 to 9.3 mg/100 ml), in agreement with Webster *et al.*(10). On the other hand, gastric 'mucoproteose', which is known to be derived from the surface epithelium(17,18) was present in all these collections, its concentration ranging from 15.9 to 62.1 mg (mean: 31.1 mg/100 ml). These values were slightly below those obtained by Webster *et al.*(10) on the secretions of rat gastric mucosa explanted by the same method (mean: 49.2 mg/100 ml).

Our results have confirmed the fact that the explant technic in rats(9,10) achieves a situation comparable to that encountered in advanced gastric atrophy in humans(5,7,17, 18).[‡] None of the glandular products (*i.e.*, HCl, pepsin, 'cathepsin' or 'glandular mucoprotein') are present here in appreciable amounts. Only gastric 'mucoproteose' was detectable, again confirming the derivation of this material from the surface epithelium (14,17,18), a view corroborated by other investigators(10,19).

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[†] The technical assistance of Sallie E. Mitchell and Marilyn Rich in these chemical and electrophoretic studies is here gratefully acknowledged.

[‡] A similar dedifferentiation and atrophy of the gastric mucosa has recently been reported in dogs, with use of the exteriorization technic(25).

TABLE I. Composition of Rat Gastric Juices and Secretions from Explanted Rat Gastric Mucosa.

| | No. | No. of rats | Total vol, ml | Free acid | Pepsin, $PU_{Hb} \times 10^{-4}$/ml | Cathepsin, $CU_{Hb} \times 10^{-4}$/ml | Glandular muco-protein, mg/100 ml | Muco-proteose, mg/100 ml |
|---|---|---|---|---|---|---|---|---|
| Pooled gastric juices* | 1 | 7 | 20 | Present | 19.9 | | | |
| | 2 | 8 | 22 | " | 12.5 | | | |
| | 3 | 4 | 14 | " | 9.4 | | | |
| | 4 | 6 | 16 | " | 7.5 | | | |
| | 5 | 5 | 15 | " | 6.7 | | | |
| Secretions of gastric mucosal explants† | A-1 | 1 | 11.4 | Absent | 0 | 0 | 1.3 | 22.9 |
| | 2 | 1 | 12.0 | " | 0 | 0 | 9.8 | 63.7 |
| | 3 | 1 | 10.0 | " | 0 | 0 | 4.0 | 28.0 |
| | 4 | 1 | 13.0 | " | 0 | 0 | 4.4 | 21.2 |
| | 5 | 1 | 12.2 | " | 0 | 0 | 5.5 | 72.1 |
| | 6 | 1 | 10.0 | " | 0 | 0 | 2.6 | 25.4 |
| | B-1 | 1 | 2.5 | " | 0 | 0 | 0 | 15.9 |
| | 3 | 1 | 2.0 | " | 0 | 0 | 0 | 19.9 |
| | 4 | 1 | 2.8 | " | 0 | 0 | 0 | 19.1 |
| | 5 | 1 | 4.2 | " | 0 | 0 | trace | 25.5 |
| | 6 | 1 | 3.5 | " | 0 | 0 | 0 | 28.5 |

* Each pool was obtained by pooling the 24-hr secretions of stomachs, ligated at cardia and pylorus, of from 4 to 8 rats.

† Secretion from each rat, A-1 to A-6, represents total of 10-12 continuous collections from the explant over a period of one month. Secretions from rats B-1 to B-6 represent materials collected over 5-7 days.

*Paper-electrophoretic analysis of explant secretions.* Electrophoretic patterns of normal rat gastric juice (Fig. 1) are similar to those of humans (Fig. 2) except for components Y and Z, which, in rat juice, are either absent or present in very low concen-

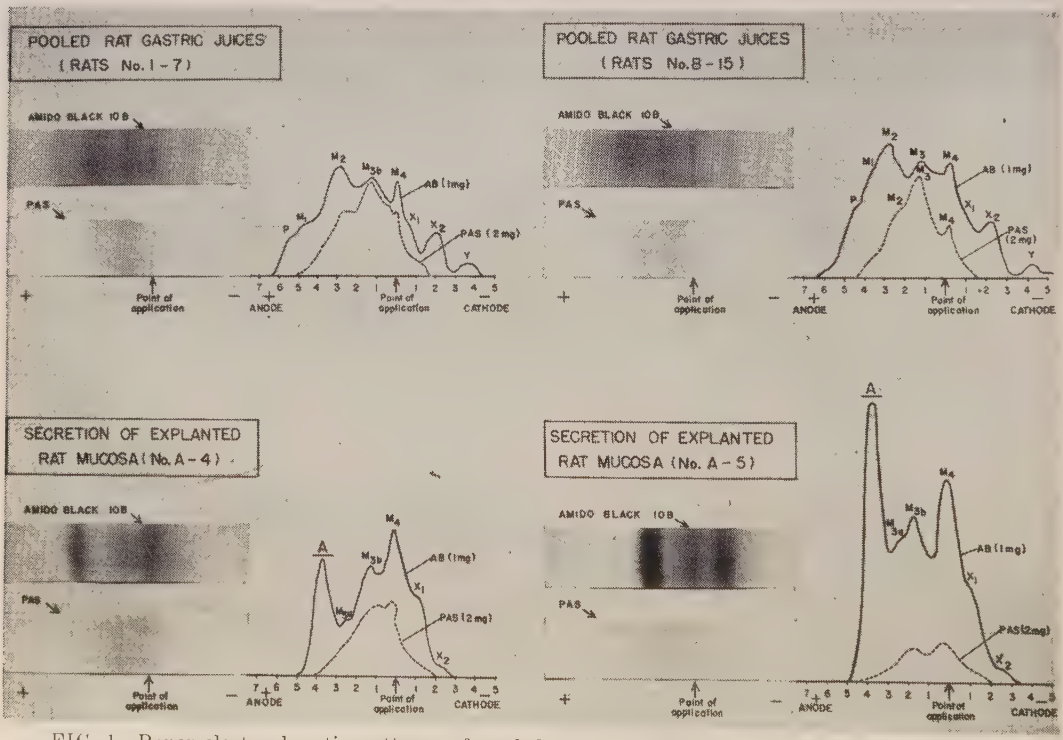

FIG. 1. Paper electrophoretic patterns of pooled normal gastric juices and secretions from explanted gastric mucosa of rats. (Amido black 10B and PAS stains.)

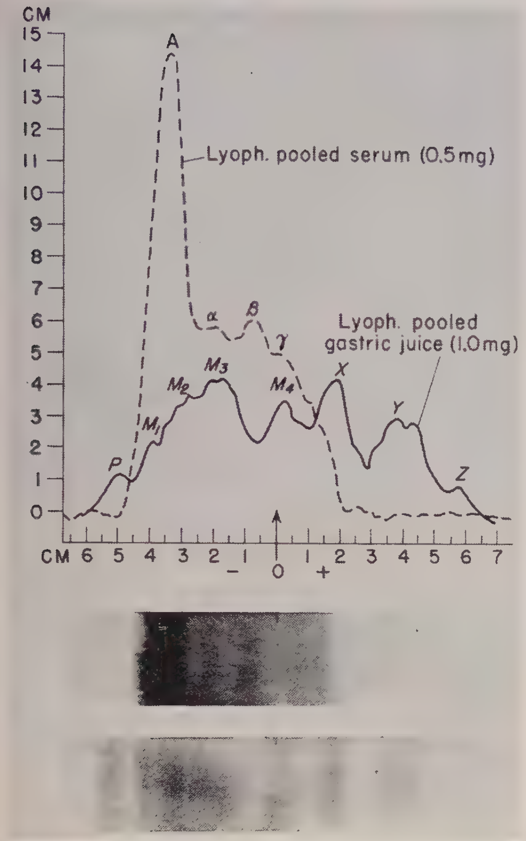

FIG. 2. Paper electrophoretic patterns of normal gastric juice and serum in man. (Amido black 10B stain.)

tration. Electrophoregrams of the secretion from the explanted mucosa differ markedly, however, from those of the normal rat gastric juice by absence of pepsin (P), glandular mucoprotein ($M_1$), the cathodic component $X_2$, and by high concentration of components $M_3$ and $M_4$ (Fig. 1). This agrees with the findings of Webster *et al.*(10) and others, and with the data given above. Further, these electrophoretic studies point again to the surface epithelial origin of gastric mucoproteose (14,17-19) and its electrophoretic correlates, components $M_3$ and $M_4$ (2,3,6).

The most marked abnormality of the secretion from rat explanted mucosa is the presence of a large component, labelled "A", which has a mobility similar to that of mucous substance $M_2$, but differs from it by staining heavily with amido black 10B and only slightly with carbohydrate-staining PAS (Fig. 1). Identical patterns were obtained for other explants (Fig. 3). This component has the electrophoretic mobility and staining properties of serum albumin as shown by Fig. 2 which includes paper-electrophoretic tracings of normal human gastric juice and serum.

This albumin-like material shows other features also, reported elsewhere(20,21) which speak for its identity with serum albu-

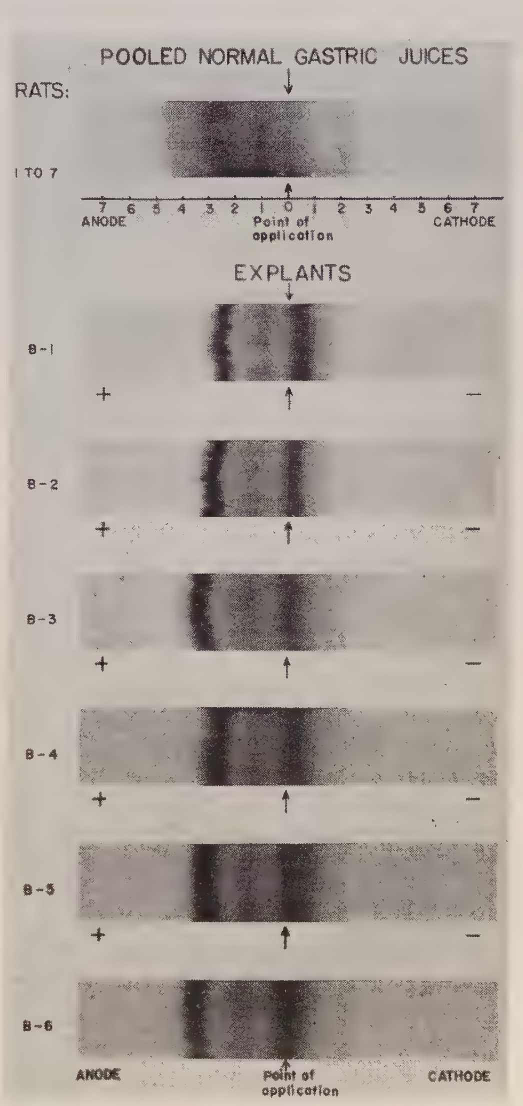

FIG. 3. Paper electrophoretic patterns of pooled normal gastric juices of 7 rats and of 6 explants of rat gastric mucosa. (Amido black 10B stain; 0.4 mg of dialyzed and lyophilized materials applied to the center of the strips.)

min. These are: 1) Precipitability with trichloracetic acid. 2) An electrophoretic mobility of the trichloracetic acid precipitate identical to that of serum albumin at pH 7 to 9. 3) Solubility of this trichloracetic acid precipitate in 80% acetone and 95% alcohol, a unique feature of serum albumin precipitate.

In many electrophoregrams of explant secretions an additional component may be detected with amido black 10B (Fig. 1) and SF light green (not shown in Figure), but which stains very little with PAS and is located slightly to the cathodic side of the application point in the area of the $M_4$ band. This component probably represents gamma globulin which has passed (from the blood serum) into the secretion of the mucosal explant.

*Discussion.* Secretions from the explanted rat gastric mucosa are electrophoretically similar to those of the gastric juices of patients with protein-losing gastropathies. We have previously reported similar electrophoretic tracings in the anacid gastric juices of patients with gastric cancer, atrophic gastritis, pernicious anemia and Menetrier's disease(6, 8,20). Secretions from all the rat explants show a similar albumin-like band. Most of them also contain a large component in the area of the $M_4$ band which probably represents serum gamma globulin. The presence of these serum protein-like materials in secretions of the explanted mucosa is not due to an admixture of blood, as proven by its absence from collections and no hemoglobin band in the electrophoregram of the gastric juice, the usual location of which is, with our technic, slightly to the anode from the application point.

The explanted rat gastric mucosa probably leaks serum proteins in a way similar to that of the gastric mucosa of patients with protein-losing gastropathies(22,23). Leakage of serum proteins through the explanted gastric mucosa of rats may be due both to mucosal atrophy(6) and ulcerations which exude serum proteins. The absence of acid and pepsin in these secretions prevents degradation of albumin, thus making its detection possible in the electrophoretic tracing(8,21,24).

*Summary and conclusions.* Gastric secretions were collected from the stomachs of rats with ligated cardia and pylorus and from the explanted gastric flaps of male rats. Explantation of gastric flaps in male rats results in atrophy of the superficial elements of the gastric glands, obliteration of glandular ducts by overgrowth of cuboidal surface epithelium, and fibrosis and superficial ulceration of the explanted mucosa.

The absence of all glandular components of gastric juice from the secretion of the explants, as previously reported by other workers, was confirmed. This was true of HCl, pepsin, 'cathepsin' and 'glandular mucoprotein' and their electrophoretic correlates, components P and $M_1$, as well as of the cathodic component $X_2$. Conversely, the presence of gastric mucoproteose and, on paper electrophoresis, the corresponding electrophoretic band $M_3$, confirms the surface epithelial origin of these materials. The electrophoretic patterns of secretions from explanted gastric mucosa are similar to those observed in gastric juices of patients with protein-losing gastropathies in that they contain a serum albumin-like component which stains heavily with amido black 10B and SF light green stains, but not with PAS. The presence of serum protein-like materials in explant secretions is not due to an admixture of blood, but is probably due to transudation of serum proteins through the atrophic mucosa, or exudation of serum from superficial ulcerations which have developed in the explanted mucosa, or to both of these factors.

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Received Feb. 2, 1961. P.S.E.B.M., 1961, v107.

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## A Method for Obtaining Monolayer Cultures of Human Fetal Cells from Term Placentas.* (26689)

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The human placenta at term is considered by most authorities to be a senescent organ and histological preparations show extensive fibrinoid degeneration, calcification and infarction(1,2,3,4). In previous years investigators have reported failures in their attempts to grow cells from cultures of the villi of mature placentas(5,6). More recently Stewart, Sano and Montgomery(7), using explant cultures, obtained an outgrowth of fibroblasts from the villous portion of the mature placenta, but they were unable to grow the trophoblast or to detect in the growth medium any of the gonadotrophic hormone that is produced by the trophoblast *in vivo*. However, we found, while studying cultures of cells from placentas procured at time of therapeutic abortion, that it is also possible to obtain vigorous healthy cultures of a mixed cell type from the villi of mature placentas. In some cases chorionic gonadotrophin was demonstrated. Because of the shortage of human embryonic tissue for investigative work, the possibility that the villous portion of the mature placenta might provide an additional source of fetal cells was further explored. This paper describes a uniformly reliable technic for obtaining monolayer cultures of human fetal cells from the chorionic villi of mature placentas and describes the methods used to identify these cells as being of fetal origin.

*Methods and materials.* (A) *Tissue culture.* Mature placentas from 14 male and female pregnancies were collected aseptically at birth. Most of these were delivered vaginally. Individual cotyledons were identified on the maternal surface, excised and placed on a sterile towel with the fetal surface up. The membranes were removed exposing fetal tissue, and a layer containing chorionic villi

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* This investigation was supported jointly by the Richard W. Goode and Mae Stone Goode Gift and by USPHS grant DGMS-RG7337.

† U.S.P.H.S. Post-doctoral Fellow.

5 to 8 mm thick was dissected from the central portion of each cotyledon. This material was pooled and an aggregate of approximately 25 g was washed 5 times in saline D2(8) to remove the blood and maternal elements. Random samples of these washed villi were taken for histological examination. The washed tissue was then transferred to a 250 ml Erlenmeyer flask with 45 ml of 0.03% trypsin (Nutritional Biochemical 1:300) in saline D2 adjusted to pH 7.0 and vigorously agitated at 37°C by means of a teflon-covered magnet over an insulated magnetic mixer. When the solution became turbid aliquots were taken for cell counts every 15 minutes until most of the cells were found to occur singly or in very small clumps. Length of trypsinization varied from 45-105 minutes. The suspension was then filtered into a beaker covered by a single layer of lint-free gauze. All of the tissue residue was recovered from the gauze and fixed for histological sections. The filtrate was centrifuged in a 50 ml round bottom tube at 800 rpm for 8 minutes and the supernate discarded. The spun cell sediment was suspended in 30 ml of saline D2 by gentle pipetting and a cell count made. The cell suspension was centrifuged again and resuspended in growth medium to a final concentration of $4.5 \times 10^5$ cells per ml. Growth medium consisted of 80% NCTC 109‡ and 20% fetal bovine serum§ with penicillin 100 units/ml and streptomycin 50 gamma/ml. Each $16 \times 150$ mm screw-capped roller tube containing a flying coverslip $6 \times 30 \times 0.2$ mm was inoculated with 2 ml of the cell suspension and incubated in a slanted stationary position at 37°C in a gassed incubator with 7% $CO_2$ in atmospheric air at 80% relative humidity. An average of 25-30 roller tube cultures was obtained with this technic. By removing larger amounts of tissue from the cotyledons, more cultures could be obtained. The medium was completely changed after 24 hours and then twice a week. After the first nutrient change, the medium removed was quantitatively assayed for chorionic gonadotrophin after the method of Delfs(9). The cultures, which were examined every other day, became confluent and ready for subculture in 10-14 days. (B) *Subculture method.* The growth medium was removed and the cells washed in saline D2 for 2-3 minutes. This was discarded. Each culture was washed again for 15-25 minutes at room temperature with 2 ml of saline D2 until the sheeted cells floated off the glass, at which time 2 drops of 0.25% trypsin in saline D2 were added to each roller tube. With gentle agitation a single cell suspension was obtained in 5 minutes. After a count was made of the pooled cell suspension, it was centrifuged. The spun cells were resuspended in growth medium to a final concentration of $4.5 \times 10^5$ cells/ml and roller tube subcultures made. (C) *Staining.* The samples of washed villi and the tissue residue after trypsinization were fixed in modified Davidson's solution(10) for 24 hours and then remained in 70% ethyl alcohol until paraffin sections were prepared and stained with hematoxylin and eosin. The flying coverslips were removed at random intervals during the first 10 days. Some were fixed in absolute methyl alcohol and stained with May-Grunwald and Giemsa to define cellular morphology. The remaining coverslips were stained to demonstrate sex chromatin. These were fixed in equal parts of 95% ethyl alcohol and ether, hydrolyzed in N HCl and stained in buffered thionin after the method described by Klinger(10). A quantitative analysis for sex chromatin was made of the primary cultures from each placenta by counting at least 200 interphase nuclei in each preparation. Overlapping nuclei or those showing degenerative changes were omitted. All other nuclei with a chromocenter were counted as sex chromatin positive. All the counts were made on primary cultures less than 2 weeks old because of reports that the number of chromatin positive nuclei decreases with subculturing(11). (D) *Controls.* Normal adult female buccal smears were used to insure the effectiveness of the thionin stain. The maternal placental surface of 2 male gestations, cultured in the manner described for the fetal surface, was used as a control to reflect the changes in the

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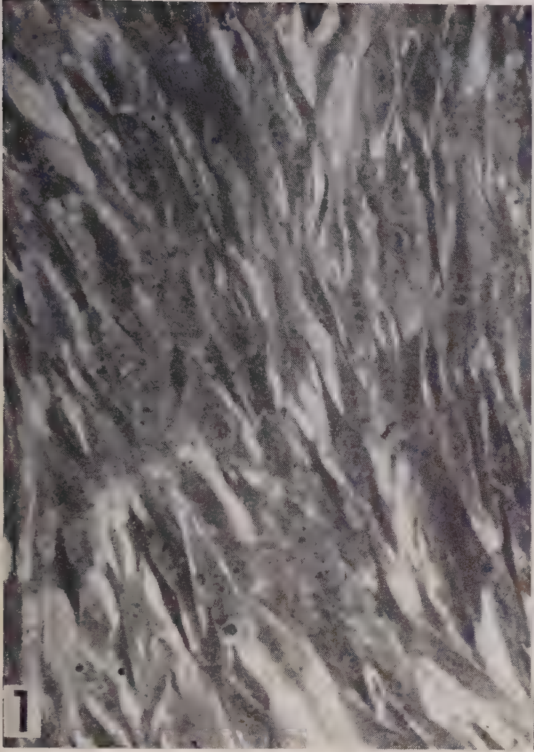
‡ Microbiological Associates, Bethesda, Md.

§ Colorado Serum Co., Denver.



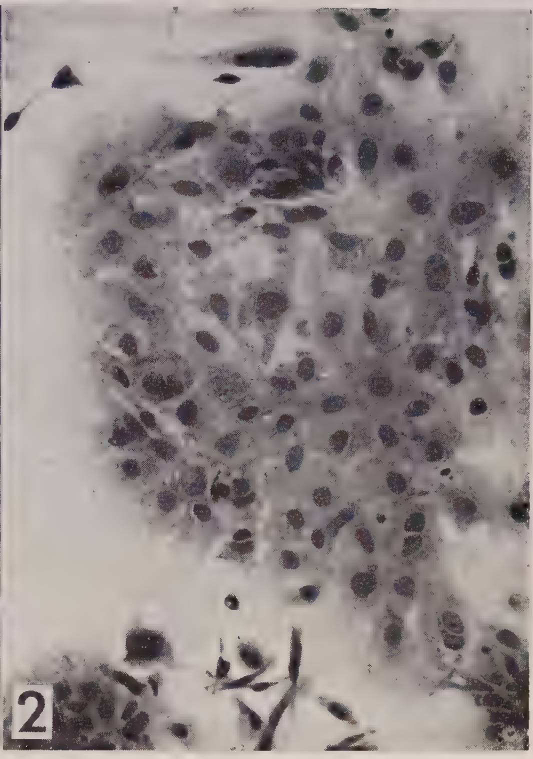


FIG. 1. A sheet of spindle cells. FIG. 2. A typical colony of epithelioid cells found in a monolayer culture of fetal placental cells from a mature placenta.

percentage of sex chromatin positive cells due to presence of decidual tissue.

*Results.* A total of 16 term placentas were processed for tissue culture. Fourteen were succesfully maintained in prolonged culture. Delay in obtaining one placenta after delivery and prolonged trypsinization of the other explained the 2 failures. Bacterial contamination was not a problem. The primary cultures of the fetal placental tissue showed a mixed cell population indistinguishable from the cell types previously described with cultures of therapeutic abortion material (12). These were (a) large and small spindle shaped cells (Fig. 1), (b) clusters of epithelioid cells which grew in a pavement like fashion (Fig. 2) and (c) occasional multinucleated giant cells. Following the first subculture, the spindle cells became the predominant cell and only a few colonies of epithelioid cells were seen. Chorionic gonodatropin hormone (HCG) was detected in the culture medium of 2 term placentas using the immature rat uterine weight method described by Delfs(9). Each ml of ether extracted medium (EEM) from one term placenta (TP I) after 3 days in culture contained 62.5 International Units (IU) of HCG. At 7 days none was found. The medium from TP III after 3 days in culture contained 277 IU of HCG per ml EEM and after 5 days in culture 700 IU of HCG per ml EEM. At 7 days and 10 days the assays were negative. These data suggest an *in vitro* production of HCG by the TP III cells, but this is not regarded as conclusive evidence. Careful examination of the histological sections of washed fetal placental tissue revealed no decidua. Multiple sections from the trypsinized fetal tissue of each placenta were also searched for decidua. Rarely was any found except for 3 specimens in which many single decidual cells and several small clumps of decidua were present. The decidual cells were clearly defined as large round intensely eosinophic cells with round nuclei containing a single prominent centrally located nucleolus. The percentage of nuclei containing chromocenters was calculated for each placental culture. Less than 5% sex chromatin positive nuclei were found in all but 3 of the male fetal placental cell cultures. The counts of those 3 were 8%, 14% and 19% positive nuclei. The significance of this will be discussed. An average of 50% of the nuclei in the female fetal placental cell cultures contained sex chromatin. Examination of the histological sections of the washed and trypsinized tissue taken from the maternal surface of 2 male placentas revealed a somewhat greater number of decidual cells. An average of 35% of these cells in culture contained sex chromatin positive nuclei.

*Discussion.* The human placenta is largely composed of fetal tissue supported on a framework of maternal cells. The small amount of maternal tissue can be avoided by careful dissection. To confirm the fetal origin of the cell cultures obtained from the chorionic villi and to quantitate the number of maternal cells inadvertantly included, the sex chromatin pattern of the cell cultures was studied. Previous investigators have shown that the cellular elements of the fetal portion of the placenta have a sex chromatin pattern identical with the morphological sex of the fetus(13,14,15). Others report that the nuclei of the cells in the placental septum have a female chromatin pattern regardless of the sex of the fetus(16). The results of our studies agree with these reports. Fetal placental cell cultures from 11 male gestations contained less than 5% sex chromatin positive nuclei. This falls within the accepted percentage of male nuclei which contained chromocenters(17). Decidual cells were rare in the trypsinized material from which these cultures were obtained. The 3 fetal placental cell cultures which contained more than 5% sex chromatin positive nuclei were obtained from the same specimens in which greater numbers of decidual cells were found in the histological sections. Cultures of cells taken from the maternal surface of the placentas of male gestations also contained a greater number of nuclei with a female chromatin pattern. Histological sections of the trypsinized material showed a proportionate increase in decidua. It is clearly evident that with improved methods and materials, it is now possible to obtain human fetal cell cultures from the chorionic villi of mature placentas. The sex chromatin studies of the placental cell cultures from male gestations confirm the fetal origin of the cells. Placentas from both male and female gestations can be used to obtain these fetal cell cultures. Although sex chromatin studies are of no value in estimating the amount of decidua in placental cell cultures of female gestations, examination of histological sections of the trypsinized material will detect the inadvertant inclusion of maternal elements. These fetal cells provide investigators with an additional source of human embryonic tissues for experimental studies using tissue cultures.

*Summary.* Technics are presented for obtaining monolayer cultures of human fetal cells from the chorionic villi of mature placentas. Mixed cultures of epithelioid and spindle cells are obtained with these methods. The fetal origin of the cells in culture is confirmed with studies of the sex chromatin pattern of the nuclei and by examination of histological sections of the trypsinized tissue for the presence of decidua. Detection of chorionic gonadotrophin in the medium of 2 cultures of cells from mature placentas is reported.

The investigators are indebted to Dr. Herbert R. Morgan for consultation and advice during these studies and to Virginia Eldridge and Eleanor Dudley for technical assistance.

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Received April 24, 1961. P.S.E.B.M., 1961, v107.

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# Dissociation of Uridine Diphosphate Glucose-Glycogen Transglucosylase from Phosphorylase Activity in Individual Muscle Fibers. (26690)

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The present concept of a cyclic glycogen metabolism in muscle(1) assumes the existence of different enzymatic pathways for glycogen synthesis and breakdown. The catabolic enzyme is represented by phosphorylase(2), and polysaccharide synthesis from glucose-1-phosphate is considered to depend on the uridine diphosphate glucose (UDPG)-linked pathway with the UDPG-glycogen transglucosylase reaction(3) as the final step.

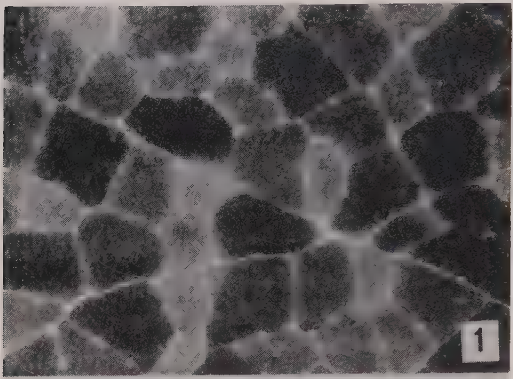
Histochemical studies on the distribution pattern of these enzymes in resting skeletal muscle of the rat revealed a reciprocal relationship between phosphorylase and UDPG-glycogen transglucosylase activity in individual fibers. A different spatial arrangement of the two activities would be inconsistent with their operating as parts of a single enzymatic cycle.

*Methods.* Muscle tissue, excised from rats bled under ether anesthesia, was quenched in liquid oxygen. From tissue blocks oriented in a transverse or longitudinal plane fresh frozen sections were cut at 8 $\mu$ on a cold microtome (cryostat) at –20° and mounted on coverslips. The activity of phosphorylase, branching enzyme (amylo-1, 4 → 1, 6-transglucosidase) and UDPG-glycogen synthesizing enzyme was demonstrated according to Takeuchi(4,5,6). The reaction for the last of these was carried out in the presence of .0005*M* glucose-6-phosphate. The glycogen content of untreated cryostat sections was assessed by the periodic acid-Schiff procedure after post-fixing the sections in Gendre's fluid. The methods employed for demonstration of various oxidative enzymes have been listed previously(7).

*Results.* A striking difference in enzymatic activity was noted in longitudinal and cross sections of muscles containing fibers of varying size. Both phosphorylase and branching enzyme were strongly active in fibers of large diameter(7). Conversely, high activity of UDGP-glycogen-transglucosylase was obtained in fibers belonging to the small ("red") type (Fig. 1, 2). Fibers of intermediate size showed varying degrees of activity with either type of reaction. Strong UDGP glycogen-transglucosylase activity was accompanied by high activity of oxidative enzymes, especially succinate and lactate dehydrogenases. In striated muscle of uniform fiber type (for instance, tongue, *M. levator ani*) little or no variation in enzyme activities was observed.

In contrast to findings in pigeon breast muscle(8) resting rat muscle showed intrinsic, diastase-labile, glycogen to be concentrated in the narrow fibers. In the same fiber type, polysaccharide formation was dependent mainly on the UDGP-transferase reaction.

The phosphorylase reaction was inhibited by substituting adenosine-5′-triphosphate



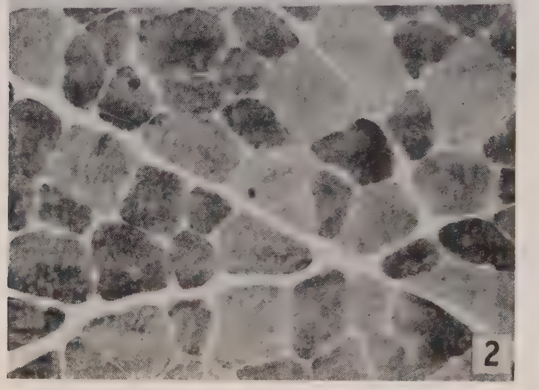


FIG. 1. M. rectus femoris of the rat. Phosphorylase. 170 ×.

FIG. 2. Serial section to Fig. 1. Reaction for UDPG-glycogen transglucosylase. 170 ×.

(ATP), adenosine-5′-diphosphate (ADP) or cyclic adenosine-3′,5′-phosphate for 5′-adenylic acid (AMP) in the medium. No influence of these nucleotides on glycogen formation from UDPG was observed. Addition of glucose or sodium-$\beta$-glycerophosphate (0.05M) caused marked reduction in polysaccharide formed from glucose-1-phosphate, but inhibited the UDPG reaction only slightly. Under the influence of inhibitors, no change in distribution pattern of the reaction products was observed.

*Discussion.* The reciprocal localization of the 2 possible metabolic routes from glucose-1-phosphate to glycogen (the phosphorylase and branching enzyme reaction on one hand and the UDPG-pathway on the other) in individual muscle fibers lends further support to the view that the contractile function of the red and white fibers of mammalian muscle depends on fundamentally different metabolic systems(9). The main reason for the observed difference may be a marked variation in levels of phosphorylase activity between individual fibers. Polysaccharide may, in fact, be formed from UDPG in broad fibers but escape detection because it may be broken down rapidly by the phosphorylase present in excess. If this were so, low demonstrable glycogen would not necessarily indicate actual low levels of UDPG enzyme. In resting muscle, low phosphorylase in the small fibers was accompanied by both high levels of intrinsic glycogen and glycogen synthesis from UDPG.

A catabolic action of demonstrable phosphorylase on polysaccharide formed from UDPG would fail to explain why, on the other hand, high activity of both enzymes can be shown histochemically in a single structure as, for instance, in heart muscle and in skeletal muscle of a uniform fiber type. Apparently, the levels of activity of enzymes acting on glycogen are of importance(10).

ATP, which has no influence on partially purified UDPG-glycogen transglucosylase (3), did not inhibit polysaccharide formation from UDPG in the histochemical reaction. If activation of catabolic phosphorylase *a* by ATP(11) takes place in tissue sections, the failure of the nucleotide to induce breakdown of glycogen synthesized from UDPG could indicate either very low levels of phosphorylase in small fibers or that phosphorylase is not capable of degrading the newly formed polysaccharide. The latter seems unlikely since both enzymes are presumed to act on 1,4-linkages of the glucose polymer(1). Histochemical polysaccharide formation from glucose-1-phosphate is dependent on the presence of AMP which is a cofactor required by phosphorylase *b*(12); ATP cannot substitute for AMP, and in the presence of ATP the histochemical reaction is inhibited.

An alternative explanation for the observed differences in activity of individual fibers would be the assumption of 2 separate polysaccharide forming mechanisms in muscle, one *via* phosphorylase and the other *via* the uridine linked pathway. The discussion of this possibility would require more evidence on the nature of the histochemical reaction than is available. The most promising approach would appear to be the study of tissues known to be deficient in enzymes for glycogen utilization(13).

*Summary.* A reciprocal relationship between phosphorylase and UDPG-glycogen transglucosylase activity has been demonstrated by a histochemical technic in individual fibers of resting rat skeletal muscle. In general, polysaccharide formed from glucose-1-phosphate was deposited in the large (white) type of fiber and that from UDPG in the small (red) fibers.

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Received March 21, 1961. P.S.E.B.M., 1961, v107.

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## Effect of Adrenalectomy and Successive Hydrocortisone Replacement on Thyroid Secretion Rate in Male and Female Rats.[*] (26691)

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It has been suggested that adrenalectomy decreases thyroid function as a consequence of decreased metabolism(1), while other studies concerning exogenous administration of adrenocorticoids to animals have shown the following effects: a suppressive action of TSH release in normal rats(2,3); a stimulating action on TSH release in normal rats(4); a direct suppression on thyroid gland in hypophysectomized rats(5), normal rats(6) and normal chickens(7); reduction in peripheral utilization of thyroxine in normal rats(8); no effect in normal dogs(9); and a stimulating effect on thyroid gland in normal rats(10).

Determination of thyroxine secretion rate (TSR) has not been used to evaluate adrenocortical effects on thyroid function. Previous indices were $I^{131}$ uptake and release and thyroid cell height. With accurate technics for determining TSR in rats available(11), present work was undertaken to determine TSR in individual animals before and after adrenalectomy and after subsequent adrenocortical replacement.

*Materials and methods.* Mature male (34) and female (25) albino rats of Sprague-Dawley-Rolfsmeyer strain were used. They were fed Purina lab chow *ad libitum* and were maintained at a constant temperature of 78 ± 1°F. Initial body weights ranged from 200 to 460 g.

TSR determinations were made with use of $I^{131}$ by method of Pipes and Turner(12). Increments of 0.25 $\mu$g L-thyroxine ($T_4$) were used. Adrenalectomy (ADRX) was performed after normal secretion rates were obtained. Animals were maintained on 1% NaCl drinking water after ADRX and throughout glucocorticoid replacement therapy. Determination of TSR was commenced 2 weeks after ADRX. Replacement therapy

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[*] Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2287. Approved by Director. Aided in part by research grants from P.H.S. and U.S.A.E.C.

[†] Predoctoral fellow of N.I.H.

TABLE I. Effect of Adrenalectomy and Replacement Therapy on Thyroxine Secretion Rate of Rats (TSR in μg/100 g BW/Day).

| | Normal | | | 2 wk after adrenalectomy | | | 2 wk after hydrocortisone replacement (150 μg/day) | | |
|---|---|---|---|---|---|---|---|---|---|
| Phase | Sex | No. | TSR | Sex | No. | TSR | Sex | No. | TSR |
| Intact (1st phase) | ♂ | 34 | .93 ± .05 | | | | | | |
| | ♀ | 25 | .97 ± .06 | | | | | | |
| | Both | 59 | .94 ± .04 | | | | | | |
| Survivors, 2 wk after adrenalectomy (2nd phase) | ♂ | 18 | .90 ± .06 | ♂ | 18 | .75 ± .05* | | | |
| | ♀ | 21 | .98 ± .06 | ♀ | 21 | .67 ± .04† | | | |
| | Both | 39 | .94 ± .04 | Both | 39 | .71 ± .03† | | | |
| Survivors, 2 wk after hydrocortisone replacement of 150 μg/day (3rd phase) | ♂ | 10 | 1.00 ± .07 | ♂ | 10 | .83 ± .05 | ♂ | 10 | 1.05 ± .05 |
| | ♀ | 15 | 1.02 ± .07 | ♀ | 15 | .65 ± .05† | ♀ | 15 | 1.13 ± .06 |
| | Both | 25 | 1.01 ± .05 | Both | 25 | .72 ± .04† | Both | 25 | 1.10 ± .04 |

Significantly different from normal at: * 5% level of probability; † 1% level of probability.

of 150 μg hydrocortisone acetate/animal/day was given subcutaneously in 0.15 ml saline and gum arabic suspension.

Replacement therapy was commenced when TSR determination following ADRX was completed. It was continued through determination of third TSR, which was begun 2 weeks after start of hydrocortisone replacement.

*Results.* Original 34 normal male rats had average TSR of 0.93 ± 0.05 μg/100 g BW/day while 25 normal females had TSR of 0.97 ± 0.06 μg (Table I). No significant difference between sexes was shown. After ADRX 18 males that survived had TSR of 0.75 ± 0.05 μg, a decrease of 17%, while 21 surviving females averaged 0.67 ± 0.04 μg, a decrease of 32%. Reduction of TSR in males after ADRX was significant at 5% level from their normal control values, while reduction of TSR in females was significant at 1% level. At end of third TSR determination, which was started 2 weeks after replacement with 150 μg hydrocortisone/day, values on 10 remaining males averaged 1.05 ± 0.05 μg and on 15 females averaged 1.13 ± 0.06 μg. Although these values were slightly higher than their normal control values, no significant differences were found. Body weight was not affected by ADRX or hydrocortisone replacement.

*Discussion.* These data indicate that no sex differences are found in normal TSR of mature rats of this strain. ADRX caused significant reduction in TSR when an interval of 2 weeks was allowed for equilibration of thyroid with existing metabolic state. Recent work has indicated that underfeeding causes significant reduction in TSR of intact mice(13). Furthermore, ADRX is accompanied by marked reduction in feed consumption(14). Other work has shown that ADRX animals receiving saline consumed feed at a lower level than normal(15). Our own data on 6 mature male rats revealed that feed consumption was reduced significantly from a normal level of 14.9 ± .8 g/day to 9.7 ± 5 g/day following ADRX and while maintained on 1% NaCl drinking water. These observations lead us to suggest that reduced TSR after ADRX may be due in part to reduced feed consumption rather than ADRX *per se.*

Replacement therapy with 150 μg hydrocortisone acetate plus 1% NaCl was sufficient to return TSR to normal. It is suggested that hydrocortisone increased metabolic rate by systemic action on all cells of body and not selectively on thyroid cells. Hydrocortisone doses ranging from 1 to 10 mg/day, used by previous investigators studying adrenocortical-thyroid interrelationships are considered to be above physiological levels and not representative of normal conditions. It is suggested that level of hydrocortisone used in present experiment represents physiological requirement.

*Summary.* L-thyroxine secretion rates (TSR) averaged 0.94 μg/100 g/day in 34 male and 25 female albino rats. No sex dif-

ferences were found. TSR determined 2 weeks after ADRX while on 1% NaCl replacement therapy was significantly reduced by 17% in males and 32% in females. TSR determined after 2 weeks of hydrocortisone replacement (150 μg/day) in addition to 1% NaCl was found to return to normal level. Body weight was not affected by treatments. It was suggested that reduced TSR after ADRX may be due, in part, to reduced feed consumption rather than ADRX *per se*.

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Received April 14, 1961. P.S.E.B.M., 1961, v107.

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# An Interferon Appearing in Cell Cultures Infected with Measles Virus.*
(26692)

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Several virus-cell systems have recently been shown to produce substances capable of inhibiting growth of related and unrelated viruses. The mode of action of these inhibitors has been clearly distinguished from interference due to viral components. Isaacs' interferon(1), Cooper and Bellett's Transmissible Interfering Component(2) and Ho and Enders' Viral Inhibitory Fluid(3) all seem to be similar. Ho and Enders(4) noticed that fluids of tissue culture, infected with the measles virus retarded growth of poliovirus and inhibited its cytopathic effect. We have been able to confirm these findings and to demonstrate that they depend upon the presence of an interferon-like agent which will be referred to subsequently as measles interferon. By this designation we do not imply that it is identical with influenza interferon as described by Isaacs and Lindemann, but only that, in our opinion, it represents one of a class of substances with similar properties. The evidence supporting this opinion will be presented.

*Materials and methods. Cell cultures.* Primary cultures of human amnion cells were prepared from trypsinized human amnion membranes. The culture medium consisted of equal parts of bovine amniotic fluid and Hanks' saline solution, enriched with 5% horse serum. This medium will be referred to as "regular BAF medium"(5).

Chick cell cultures were prepared from 9 to 10-day old chick embryos. Growth medium consisted of regular BAF medium with 2% chick embryo extract.

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* This investigation was conducted under the sponsorship of Commission on Viral Infections of Armed Forces Epidemiological Board, and was supported in part by The Surgeon General, Dept. of the Army, Washington, D. C.

† Fulbright Scholar—Supported by R. I. T. (Belgium) and Lederle International Fellowship Committee. Present address: The Rockefeller Inst., New York City.

A strain of HeLa cells, supplied by Dr. R. S. Chang,[‡] and a line of human amnion origin, WS cells, supplied by Dr. R. L. Thompson,[§] were also used. These cells were nourished with Eagle's basal medium, modified as described earlier(4), enriched with 10% calf serum. Concentration of serum was reduced to 5% when virus was added.

*Viruses.* Lines of the Edmonston strain of measles virus adapted to human amnion (5) and to chick embryo cells(7) were used. Of the former the 33rd and 34th human amnion passages were chiefly studied, and will be referred to as HA virus. Of the chick embryo adapted line, mainly the 14th and 15th chick embryo cell passages were used. This line will be referred to as CE virus.

Sindbis virus strain AR-339 was furnished by Dr. R. M. Taylor.

Type I (Brunhilde) and Type II (MEF) polioviruses were also used. Stocks of both polio and Sindbis viruses were grown in human amnion cell cultures. *Assay of virus.* Serial 10-fold dilutions of the virus suspension were prepared and 3 tube cultures were inoculated with 0.1 ml of each dilution tested. Infectivity titers are expressed as log 10 units of the TCID$_{50}$/0.1 ml.

Plaque assays of measles virus were performed in either HeLa or WS cell cultures as previously described(6). *Antisera.* Measles antiserum was obtained from a Cynomolgus monkey inoculated intracerebrally with the Edmonston strain virus(8). The complement fixing titer of this serum was 1/2048. *Complement fixation tests* were carried out by the drop method of Fulton and Dumbell as modified by Svedmyr and associates(9). *Ultracentrifugation* was performed in a Spinco L-preparative centrifuge. In all instances head #40 was used. *Assay of interferon.* Routine assays for interferon activity were done with Polio Type II or Sindbis as test agents and were carried out in human amnion cultures incubated in a roller wheel at 36.5°C. Only cultures at least 14 days old were used(10). After removal of the culture fluids, 0.5 ml of fresh regular BAF medium was added to each tube. Subsequently 0.5 ml of the fluid to be tested for interferon was added in the appropriate dilution, using 2 cultures per dilution. The challenge virus was added immediately afterwards. This challenge dose consisted of either 200 TCID$_{50}$ of poliovirus or 500 TCID$_{50}$ of Sindbis virus. At this concentration the cells of control cultures were completely destroyed 3 to 4 days after inoculation. The interferon titer was expressed as the highest dilution that at least in one culture clearly diminished the CPE. Usually the lower dilutions completely inhibited CPE, whereas at the endpoint partial CPE occurred.

*Experiments and results. Production of interferon.* By comparing the development of CPE and virus output in cultures inoculated with HA or CE measles virus, we found that, although the first cytopathic changes occurred at about the same time for both viruses, subsequently a significant difference appeared in spread of CPE as well as in amount of virus produced. These observations, to be described elsewhere in detail, led us to investigate the possibility of the emergence in the culture fluids of a substance inhibiting dissemination of infection and viral multiplication. The following is an example of several comparable experiments. Eight cultures of human amnion cells were inoculated with 100 TCID$_{50}$ of CE measles virus. Fluids were collected at several intervals, pooled, centrifuged at 30,000 rpm for 2 hours to remove infective virus and assayed for inhibitory activity against Sindbis virus in human amnion cells. The results are given in Table I. Inhibitory activity was also subsequently demonstrated in fluids of HeLa or WS cell cultures infected with the measles virus. The first signs of this activity were usually found at about the time the infective virus was released, and amount of inhibitor

TABLE I. Development of Interferon in Fluids of Cultures Infected with CE Virus.

| No. of sample | Days after inoculation | CPE at time of collection | Interferon titer |
|---|---|---|---|
| 1 | 6 | 0/± | 0 |
| 2 | 11 | ++ | 1/16 |
| 3 | 15 | +++/++++ | 1/32 |
| 4 | 18 | ++++ | 1/32 |

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[§] Sterling Winthrop Research Inst., Rensselaer, N. Y.

TABLE II. Interferon Titers of Fluids from Cultures Infected with CE Virus.

| Exp. | Type of culture | CPE at time of harvest | Measles titer at time of harvest, $TCID_{50}/0.1$ ml | Interferon titer | Challenge virus |
|---|---|---|---|---|---|
| 117 E | HeLa | +++ | 2.5 | 1/4 | $MEF_1$ |
| 157 E | " | ++++ | 3.5 | 1/16 | " |
| 172 E | " | 0 | 0 | 0 | " |
| 173 E | | + | 2.5 | 0 | |
| | | +++ | 3.25 | 1/8 | |
| | | +++/++++ | 3.25 | 1/16 | |
| 357 E | " | + | ND* | 0 | " |
| | | ++ | ND | undil. | |
| | | +++ | ND | 1/2 | |
| | | ++++ | ND | 1/2 | |
| 618 E | " | ± | ND | 1/2 | Sindbis |
| | | + | ND | 1/4 | |
| | | ++++ | ND | 1/8 | |
| 674 E | " | + | ND | 1/2 | " |
| | | ++++ | ND | 1/32 | |
| 766 E | HA | ±/+ | 2.5 | 1/2 | " |
| | | ++ | 4 | 1/8 | |
| | | +++ | 4.5 | 1/16 | |
| | | ++++ | 3.5 | 1/16, 1/32 | |

* ND = not done.

increased with advancing CPE. The results of several experiments summarized in Table II give interferon titers found in fluids of human amnion or HeLa cell cultures previously inoculated with the CE measles virus, and assayed in human amnion cell cultures.

That the inhibition of CPE also reflects a decrease in viral production is shown by the following experiment. Aliquots of HeLa cell prepared interferon were added to each of 4 human amnion cell tube cultures. As controls, 4 other cultures were supplemented with fluid derived from uninfected HeLa cell cultures. All cultures were then inoculated with 500 $TCID_{50}$ of Sindbis virus. Cytopathic effect was followed and the amount of Sindbis virus produced was determined by titrations in human amnion cell cultures. The results are given in Table III. The control cultures were completely destroyed after 3 days, and yielded virus up to $10^{5.5}$ $TCID_{50}/$ 0.1 ml. The cultures with interferon were followed over a 20-day period and did not show any sign of CPE. Some virus was reproduced in these cultures, although considerably (1000 ×) less than in control cultures. The medium was changed twice during this period, and the same amount of interferon as was present at onset of experiment was added to the fresh medium. *Mode of action.* It was demonstrated in the following 2 experiments that interferon did not inactivate the virus. A mixture of undiluted, HeLa cell prepared interferon and undiluted stock Sindbis virus was incubated at 37°C for 90 minutes. Infectious virus was then

TABLE III. Effect of Measles Interferon on Propagation of Sindbis Virus in HA Cell Cultures.*

| Hr after inoc. | Amt of virus in culture fluid: Control | Amt of virus in culture fluid: Interferon | CPE: Control | CPE: Interferon |
|---|---|---|---|---|
| 0 | 2.5 | 2.5 | 0 | No sign of CPE |
| 6 | .75 | 1.25 | 0 | |
| 12 | 3.5 | 2.75 | 0 | |
| 24 | 4.25 | 2.25 | 0 | |
| 36 | 5.25 | 3.25 | +/++ | |
| 48 | 5.5 | 2.5 | ++/+++ | |
| 60 | 5.25 | 2.75 | +++ | |
| 72 | 3.75 | 2.5 | ++++ | |
| 96 | | 1.5 | | |
| 120 | | 1.5 | | |
| 168 | | 2 | | |
| 216 | | 0 | | |
| 360 | | 0 | | |

* Cultures with and without interferon were inoculated with the same amount of Sindbis virus. At indicated intervals thereafter degree of CPE was recorded and infectivity titer of culture fluids was determined. Titers are expressed as $\log_{10}$ $TCID_{50}/0.1$ ml.

TABLE IV. Results of Varying Concentrations of Interferon and Challenge Virus.

| Amt of virus added ($ID_{50}$/0.1 ml) | Concentration of interferon in culture | | | | Control cultures |
|---|---|---|---|---|---|
| | 1/4 | 1/8 | 1/16 | 1/32 | |
| $10^6$ | 2 | 4 | 4 | 4 | 4 |
| $10^5$ | ± | 4 | 4 | 4 | 4 |
| $10^4$ | ± | ± | 4 | 4 | 4 |
| $10^3$ | 0 | 0 | ± | 1 | 4 |
| $10^2$ | 0 | 0 | 0 | 0 | 4 |

Cultures were previously incubated for 2 hr with indicated amount of interferon. Thereafter challenge virus (poliovirus Type I) was added, in 10-fold increasing concentrations. Readings of CPE taken every day. Table records reading on 3rd day after inoculation.

(4 = 100% CPE; 3 = 75% CPE, etc.).

measured by titration in human amnion cells. The endpoint was identical with that of a control mixture, indicating that no direct neutralization of the virus had occurred. In a second experiment, the virus-interferon mixture was left for one hour at 37°C and subsequently kept overnight at ± 4°C. Infectivity titrations were carried out in this experiment in chick embryo cell cultures. Again, no evidence was found for direct neutralization of the virus. It should be pointed out here that HeLa cell produced interferon does not protect chick cells against infection with Sindbis virus.

The influence of increasing amounts of test virus on degree of protection conferred by interferon was next investigated. Four different concentrations of HA cell produced interferon were tested each against 5 ten-fold increasing doses of poliovirus type I. Concentration of interferon required to inhibit the CPE varied directly with concentration of the challenge virus (Table IV). *Separation of interferon from infectious particles and complement fixing antigens.* That the inhibitory substance was obtained in a system where virus multiplication took place made it necessary to study the relationship of this inhibitor to the infectious and complement fixing properties of the virus.

Ultracentrifugation for 2 hours at 30,000 rpm was sufficient to sediment infective particles and complement fixing antigens, but did not significantly depress the inhibitory activity of the supernate. These results are compatible with the size of measles virus and CF particles, as determined by Benyesh and associates using other methods(11).

Filtration through Millipore filter membranes showed differences in filterability between infectious, complement fixing and interferon particles. Three 5 ml aliquots of HeLa cell grown CE measles were passed through Millipore filter membranes respectively of 100, 50 and 10 mμ average pore size.[‖] The pH of the fluids varied between 7.1 and 7.2. The 3 filtrates were then assayed for infectious, complement fixing and inhibitory activity. All infectivity was removed after passage through the 100 mμ membrane (Table V). The amount of complement fixing antigen passing through decreased with pore size, but some activity was left even in the filtrate of the 10 mμ membrane. In contrast the concentration of interferon remained undiminished even after passage through the membrane of smallest pore size.

To obtain further information concerning the relationship of the interferon to the virus itself, we determined the effect of measles antiserum. Equal amounts of interferon and the antiserum diluted 1/10 were mixed. In one experiment the mixtures were incubated for 2 hours at 37°C, then assayed against Sindbis virus. In a second experiment the mixtures were first incubated at 37°C and kept overnight at 4°C before assay. No effect of the antiserum on inhibitory activity was found. *Some chemical properties of measles interferon.* In view of results obtained by others with similar substances(4, 12), the possibility that measles interferon is a protein was examined by submitting it to the action of trypsin. A 30 minute exposure of interferon fluid to an equal volume of 0.25% crystalline trypsin at pH 7.4 and 37°C completely destroyed inhibitory activity. Activity of samples treated with trypsinogen remained unchanged.

Incubation with either ribonuclease or deoxyribonuclease at a concentration of $100\gamma$/

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[‖] Millipore Filter Corp., Bedford, Mass. Dishes of the VF (10 mμ) VM(50 mμ) and VC(100 mμ) filters in 47 mm diam. size were used.

TABLE V. Influence of Pore Size on Infectivity, Complement-Fixing Activity and Interferon Titer.*

| | | Infectivity (titer in PFV/0.5 ml) | Complement fixing activity | | Interferon titer |
|---|---|---|---|---|---|
| | | | Undiluted | Diluted ½ | |
| Before filtration | | 3140 | + | + | 1/8 |
| After filtration | 100 mμ pore size | 0 | + | ± | 1/8 |
| | 50 " " " | 0 | + | 0 | 1/8 |
| | 10 " " " | 0 | ± | 0 | 1/8 |

* Fluid used in this experiment was obtained from a HeLa cell bottle culture, previously inoculated with CE measles agent. Fluid was harvested at time of maximum CPE. After low speed centrifugation an equal amount of phosphate buffer was added. Filtration through Millipore membranes of indicated pore size.

ml for 30 min at 37°C had no effect.

A one hour exposure to ether (20% by volume) at room temperature did not destroy the interferon.

The interferon also proved wholly or partially resistant to treatment with fluorocarbon or chloroformoctanol. A mixture of equal parts of genetron¶ (trifluorotrichloroethane) and interferon was vigorously shaken for 5 min at room temperature, then centrifuged at 1000 rpm for 5 minutes. Assay of the aqueous phase revealed only a very slight loss of activity. After one chloroformoctanol extraction, however, only about 50% of the original activity remained in the fluid.

Heating for 2 hours at 56° had no effect on interferon titer. Stability at this temperature is in contrast to that of measles virus infectivity which is rapidly destroyed under these conditions(13). The resistance of measles interferon to various physical and chemical treatments studied is summarized in Table VI along with the behaviour of the infectious and complement fixing components of the virus under the same conditions.

*Discussion.* The data provide information on the nature and properties of an inhibitor of viral activity appearing in measles infected tissue cultures. Production and release of this inhibitor is broadly coincident with that of infective virus. Degree of inhibition varied directly with amount of virus used as challenge. The inhibitor can be distinguished from the infective unit. The data also suggest that it is smaller than the complement fixing antigen. It also differs from both these viral components by its failure to be affected by specific measles antibody.

Its mode of action remains largely unknown. Since we have shown that it does not combine directly with the test virus, it probably acts like other interferons at the cellular level.

The influence of the inhibitor on spread of infection in the system in which it is produced has not been investigated in this study. Some chemical properties of the inhibitor, however, have been defined. Digestion by trypsin shows that it is a protein, or that at least part of the activity is due to a protein. Treatment with ether or with genetron did not reduce the activity. Some loss occurred after one chloroform octanol extraction. Heating for 2 hours at 56° was without effect. These

TABLE VI. Some Physical and Chemical Properties of Measles Interferon as Compared with Those of Infectious and Complement Fixing Particles.

| | Interferon | Infectivity | Complement fixing antigen |
|---|---|---|---|
| Inact. by trypsin | + | + | 0 |
| " " RNase or DNase | 0 | 0 | 0 |
| " " ether | 0 | + | 0 |
| " " Genetron | 0 | +* | 0 |
| Inact. after 2 hr at 56° | 0 | +† | 0 |
| Sedimented after 2 hr at 100,000 g | 0 | + | + |
| Retained by pore size of 100 mμ (Millipore filter discs) | 0 | + | 0 |

* Only 10% of original activity left after one extraction.

† Inactivation not complete.

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¶ Genetron 226. Division of Allied Dye and Chemical Corp., General Chemicals, Inc. (New York) Medford, Mass.

properties may be of value in testing for its presence in materials such as blood or crude tissue extracts.

The physical and chemical properties of this agent, as well as the way it is obtained, exhibit certain similarities and differences as compared with influenza interferon(12), VIF (4) and transmissible interfering component (2,14). For example, all these factors are inactivated by trypsin and are not neutralized by specific antibody. As with measles interferon, a direct correlation between amount of inhibitor produced and extent of infection of the cell system has been shown for interferons produced with influenza(15) and vesicular stomatitis virus(2). Ho and Enders, however, found with chick-adapted Type II poliovirus that maximal yields of VIF were obtained at a stage earlier than that of maximal virus production. On the other hand, in some instances influenza interferon was produced only after virus production had passed its peak(16). Another difference lies in the ease with which influenza interferon is destroyed by ether treatment as contrasted with the resistance of measles interferon to this reagent. It is of interest in this connection that the infective component of both measles and influenza virus is ether sensitive.

In spite of these differences it is reasonable to assume that measles interferon, influenza interferon, T.I.C. and VIF may be related though not identical substances. Whatever their ultimate relationship may be, it is clear that these factors represent part of the mechanism of cellular resistance to viral infection *in vitro*. It is probable that *in vivo* as well, they prove to play a role in limitation of viral infections.

*Summary.* An inhibitor of viral activity appears in cell cultures inoculated with live measles virus. This inhibitor has been separated from the infectious particles and complement fixing antigens produced in the same cultures. It is not neutralized by measles antiserum. It does not inactivate the virus when mixed with it, but reduces the extent of cytopathic changes as well as quantity of virus produced.

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Received April 21, 1961. P.S.E.B.M., 1961, v107.

# Effect of Carbon Tetrachloride on Thioridazine Action and Metabolism.* (26693)

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In an earlier study(1) we found that thioridazine (2-methylmercapto-10-[2-(N-methyl-2-piperidyl) ethyl] phenothiazine HCl, Mellaril, TP-21 had a marked effect on hepatic hemodynamics which was dose-dependent. Before proceeding further with these studies, it seemed desirable to obtain some preliminary information regarding the metabolic aspects of this compound. Since the literature suggests that the phenothiazines have their chief biotransformation site in the liver(2,3), we have studied the effect of experimentally induced liver damage on two pharmacological actions of thioridazine. In addition we have attempted to relate these effects to the persistence of this phenothiazine in the body.

*Material and methods.* Control and liver damaged male Swiss albino mice (18-30 g) were compared as to acute lethality, hypothermia and disposition of thioridazine. Liver damage was produced by administering 1 ml/kg of $CCl_4$ (20% v/v in olive oil) subcutaneously. Control animals received olive oil alone (0.1 ml per 20 g). Two groups of mice were sacrificed 24 hours after pretreatment with $CCl_4$ or oil; sections of liver and kidneys were removed and fixed in 10% formalin. Sections stained with hematoxylin and eosin were examined for histopathologic changes.† This 24-hour treatment with $CCl_4$ was the standard method employed throughout the experiment for producing liver damage.

Acute lethality determinations were carried out on control and liver damaged animals using 25, 50, 100, 200, and 300 mg/kg (i.p.) doses of thioridazine. Ten mice were used at each dosage level. The number of deaths occurring 24, 48, and 72 hours later were recorded. $LD_{50}$ values were estimated for each time interval, using the method of Litchfield and Wilcoxon(4). Similar experiments were carried out using 80, 110, 140, 170 and 200 mg/kg (i.p.) doses of pentobarbital, Na.

The hypothermic response of mice to 10 mg/kg (i.p.) of thioridazine was assessed by measuring rectal temperatures, in groups of 5 mice, at hourly intervals, with a Yellow Springs Tele-Thermometer Model 44TK; a small animal probe was inserted to a depth of 1.5 cm. Statistical comparisons of the control *vs.* the liver damaged groups were carried out by means of Student's t-test(5).

Whole body concentrations of thioridazine were determined 4 and 18 hours after its administration (50 mg/kg, i.p.). The whole animal was homogenized in a Waring Blendor in a volume of water 4 times the animal weight. The homogenate was run through the following analytical procedure which is a modification of the method of Salzman and Brodie(2).

Two ml of tissue homogenate were added to a 50 ml glass-stoppered tube, containing 1 ml of water and about 25 mg of $NaHCO_3$. Next 10 ml of n-heptane (previously washed with 1N $H_2SO_4$,1N NaOH, and $H_2O$) containing isoamyl alcohol (2% v/v) were added. The tubes were stoppered, shaken for 10 minutes, and centrifuged. Eight ml of the organic phase were then transferred to a second tube containing 4 ml of 1N $H_2SO_4$. This tube was shaken for 10 minutes and centrifuged. The aqueous acid phase was then transferred to a cuvette and the absorption determined on a Beckman DU Spectrophotometer, set at a wavelength of 263 $m\mu$. A reagent blank was carried through the same procedure by substituting water for the mouse homogenate. Using this procedure, tissue blanks were found to be negligible and

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* Supported in part by grants from U.S.P.H.S. and from Sandoz Pharmaceuticals.

† Histopathological evaluation was made by Dr. Herbert Ichinose, Dept. of Pathology, Tulane Univ. School of Med.

TABLE I. Comparison of Median Lethal Doses of Thioridazine and Pentobarbital in Oil-Treated and $CCl_4$-Treated Mice.

| Time, hr | Oil-treated $LD_{50}$, mg/kg | $CCl_4$-treated $LD_{50}$, mg/kg |
|---|---|---|
| | Thioridazine | |
| 24 | 243 (187-316)* | 148 ( 99-224) |
| 48 | 195 (141-269) | 120 ( 83-174) |
| 72 | 148 (102-214) | 96 ( 67-138) |
| | Pentobarbital | |
| 24 | 132 (116-150) | 125 (106-135) |
| 48 | 132 (116-150) | 125 (106-135) |

* 95% confidence limits.

the O.D. readings required no corrections. After quantitation at 263 mμ absorption spectra were taken on selected samples over the range of 220-320 mμ. To assess the specificity of the method, the n-heptane phase (from the first extraction step) from several mice of a given group was pooled. Equal aliquots of this pooled extracted were then washed with one each of the following phthalate or phosphate buffers(6): pH 3.0, 5.0, 6.5, and 8.0. Then, the washed heptane phases were transferred to other extraction tubes and extracted with 1N $H_2SO_4$ as before. Thus the ratio:

$$\frac{\text{O.D. reading of } H_2SO_4 \text{ after buffer wash}}{\text{O.D. reading of } H_2SO_4 \text{ without buffer wash}}$$

gives values characteristic for thioridazine. The method is similar in principle to that employed by Fujimoto *et al.*(7). To facilitate discussion, these ratios will hereafter be referred to as "partition ratios."

*Results.* Histologically, livers from $CCl_4$-treated animals exhibited extensive central coagulative necrosis and pronounced depletion of glycogen. The kidneys, on the other hand, exhibited no cellular necrosis. Some minimal nonspecific changes in cytoplasmic staining were noted; however, similar findings were seen in kidneys from oil-treated animals. It was concluded that this dose of $CCl_4$ had marked effect on the liver but little or no effect on kidney morphology.

The lethality data are depicted in Table I. Control animals exhibited the "delayed death" phenomenon, *i.e.,* deaths occurred over a period of 72 hours after administration of thioridazine. The number of "delayed deaths" was sufficient to cause progressive lowering of the $LD_{50}$ estimate through this period; the 72-hour value was significantly lower than the 24-hour value. It can be seen that $CCl_4$ treatment increases the lethal potency of thioridazine in that at all time intervals $LD_{50}$ values for the $CCl_4$-treated groups were lower than in the respective oil-treated controls; this difference was greatest at 24 hours ($p<.05$). Although $LD_{50}$ is reduced by $CCl_4$ treatment, this procedure does not seem to alter the occurrence of "delayed deaths." All $LD_{50}$ curves were found to be mutually parallel.

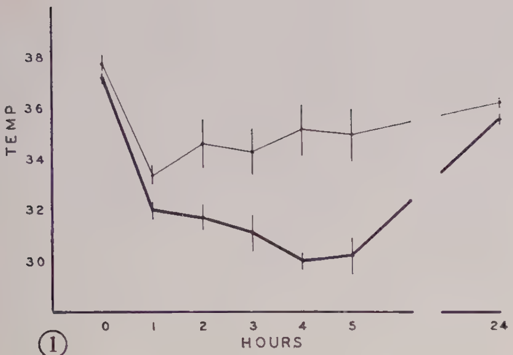
In the lower part of Table I data are included for comparative purposes, showing that $CCl_4$ treatment did not alter 24- and 48-hour acute $LD_{50}$ values for pentobarbital. These animals however did show pronounced prolongation of sleeping time. The temporal decline in the $LD_{50}$ estimate observed with the phenothiazine was not demonstrated with this particular barbiturate.

Fig. 1 illustrates the results obtained in

TABLE II. Partition Ratios of Ultraviolet Absorbing Material Recovered from Whole Body Homogenates of Mice Receiving Thioridazine.

| Group | Optical density ratio and stand. error after buffer equilibration* pH 8.0 | pH 6.5 | pH 5.0 | pH 3.0 |
|---|---|---|---|---|
| Thioridazine added to whole body homogenate *in vitro* | .80 ± .03 | .66 ± .03 | .50 ± .03 | .30 ± .03 |
| Whole body homogenate from oil-treated mice receiving thioridazine | 1.04† ± .06 | .78 ± .09 | .61 ± .08 | .39 ± .03 |
| Whole body homogenate from $CCl_4$-treated mice receiving thioridazine | .94 ± .08 | .61 ± .06 | .45 ± .05 | .33 ± .01 |

* Each value represents mean of 3 determinations.
† Significantly different from control value ($P<.05$).



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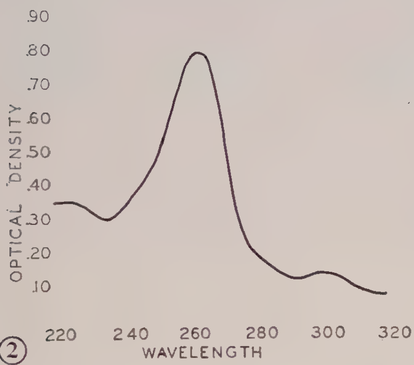

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FIG. 1. Effect of $CCl_4$ treatment on hypothermia produced by thioridazine (10 mg/kg, i.p.). Ordinate is rectal temperature (°C); abscissa is time after administration of thioridazine. Upper line depicts oil-treated control animals; lower line, $CCl_4$-treated animals. Each point represents mean of 5 animals; vertical lines represent stand. errors.

FIG. 2. Ultraviolet absorption spectrum of thioridazine HCl (10 $\mu$g/ml) in 1 N $H_2SO_4$.

the hypothermic studies. $CCl_4$ treatment resulted in both enhanced lowering of body temperature and prolongation of the effect. The temperature differences between the 2 groups were statistically significant from the 2nd hour through the 5th hour, the maximum difference occurring in the 4th hour ($p < .005$).

The mean percentage recoveries of thioridazine added to water, liver, and whole animal homogenates were 87, 93, and 84% respectively when run through the extraction procedure. Fig. 2 depicts the ultraviolet absorption spectrum of thioridazine (10 $\mu$g/ml in 1N $H_2SO_4$). It is characterized by a maximum at 263 m$\mu$.

The 4-hour whole body concentration of thioridazine determined in mice with liver damage was found to be significantly higher ($p < .05$) than in the oil-treated controls (39.3 ± (SE) 2.5 mg/kg *vs* 26.4 ± 3.0 mg/kg). This was also found to be true at 18 hours (40.4 ± 0.9 mg/kg *vs* 28.4 ± 2.0 mg/kg).

Although the data suggest that no disappearance of thioridazine occurred between 4 and 18 hours, it should be borne in mind that these results were obtained in separate experiments employing different batches of mice and were designed only to show the effect of liver damage. In another experiment using a single batch of mice designed to study the time-disappearance relationship of this drug in normal mice, it was found that mean whole body concentrations were 46.8, 25.9, and 17.9 mg/kg at 1, 4, and 18 hours respectively. Since in all cases the initial dose administered was 50 mg/kg, these data demonstrate that thioridazine, or metabolic products which are indistinguishable from it on the basis of ultraviolet spectrum, persist in the body for a relatively long time.

Mean partition ratios, and their standard errors, for thioridazine which are pertinent for assessing specificity of the method are given in Table II. It can be seen that the products extracted from oil-treated mice deviated somewhat in their partition characteristics when compared to the *in vitro* control values. However, only the difference at pH 8 was statistically significant. This suggests that the extraction procedure may extract some metabolic products as well as parent compound. The ratios obtained from the $CCl_4$ group more closely parallel those of the parent compound, although they are not significantly different from the oil-treated group.

*Discussion.* The lethality data show that $CCl_4$ treatment increases the lethal potency of thioridazine. This suggests that the presence of liver damage delays thioridazine degradation to a point where normally sublethal doses become lethal.

The lethality results were particularly striking when the effect was compared to those obtained with pentobarbital. The response to this agent is known to be affected by liver damage(8). Indeed, in the present experiments prolonged CNS depression was noted. On the other hand, the finding that lethal potency was unaltered, for pentobarbital, was initially surprising. However, after reading the literature cited by Maynert and Van Dyke(8) indicating that liver damage increases the toxicity of certain barbiturates, it was seen that most investigators have termed prolongation of anesthesia as "toxicity" rather than estimating $LD_{50}$ values, as was done here. This phenomenon may in part be peculiar to agents which have an immediate toxic effect like pentobarbital in that benactizine prolongs pentobarbital sleeping time without altering the $LD_{50}$ of pentobarbital(9).

The reason that $CCl_4$ enhances the lethal potency of thioridazine may be related to the fact that this latter substance is long acting and produces "delayed deaths" in contrast to pentobarbital. The reason for "delayed deaths" is not clear. A number of contributory factors have been suggested by other investigators working on phenothiazines(10, 11).

The results of the hypothermia studies show that at least one pharmacological action beside lethality is intensified and prolonged by $CCl_4$ treatment. These data are highly suggestive that liver damage and subsequent delay in biotransformation produce these effects.

More directly, by comparing whole body concentrations of thioridazine from oil-treated animals with those from $CCl_4$-treated animals, it is evident that liver damage delays thioridazine degradation. This is further supported by the partition ratio data, in that those obtained from $CCl_4$-treated animals more closely approximated control values for unchanged thioridazine. Although the analytical method seems fairly specific for thioridazine, the fact that the partition ratios from the control mice do not match precisely those of the parent compound suggests that perhaps some contribution from metabolic products is occurring. Of these the more likely products would be sulfoxide and N-demethylated derivatives. The former compound should be distinguishable from the parent compound in that this modification causes a shift in the spectrum of other phenothiazines(1,12). No such shifts were found in the extracts analyzed in the present study. N-demethylated derivatives, however, have approximately the same spectrum as parent phenothiazines and could contribute to the thioridazine measured(12). This may explain why even though no shift in the spectra were found the partition ratios did not give values exactly like thioridazine.

*Summary.* $CCl_4$ treatment was found to increase both lethal and hypothermic potency of thioridazine. The dose of $CCl_4$ employed produced liver damage but no kidney damage. A method is described for determining thioridazine in tissues. Whole body concentrations of this phenothiazine were found to be significantly higher in mice treated with $CCl_4$.

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Received April 21, 1961. P.S.E.B.M., 1961, v107.

# Metabolic Reduction of 1-(*p*-Acetylbenzenesulfonyl)-3-cyclohexylurea (Acetohexamide) in Different Species. (26694)

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The metabolism of tolbutamide(1-4) and chlorpropamide(5) has been studied in man and various laboratory animals. This report is concerned with metabolism of another hypoglycemic agent, acetohexamide [1-(*p*-acetylbenzenesulfonyl)-3-cyclohexylurea]. A metabolic pathway was found that differs from those observed with the other 2 drugs.

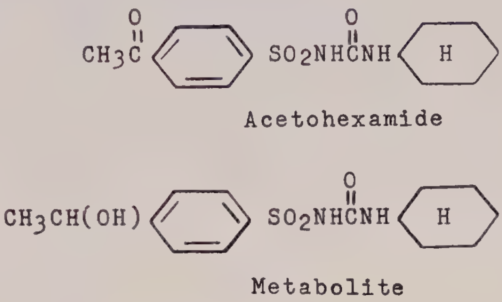

FIG. 1. Acetohexamide and metabolic product.

*Methods.* Urine specimens were acidified to pH 4 and extracted twice with equal volumes of chloroform. The combined acid-washed chloroform was reduced to 1/100 volume of urine extracted. This concentrate was spotted on Whatman No. 1 filter paper and developed by ascending chromatography with butanol saturated with 5N ammonium hydroxide. Spots were located by immersing the dried sheets in saturated mercurous nitrate, rinsing, and treating with an acetone solution of *p*-dimethylaminobenzalrhodanine, as described previously(5). For isolation of the metabolite, sheets bearing a series of urine extract spots were subjected to the above chromatographic process and a horizontal strip corresponding to the observed zone was removed. One end of each strip was placed in the solvent trough of a chromatography jar designed for descending development, and elution was achieved with water over a 24-hour period. Recrystallizations were made from aqueous alcohol. Ultraviolet absorption maxima were obtained from acidic methanol solutions (50 $\lambda$ 2N hydrochloric acid to 3.3 ml methanol). Quantitative determination of metabolite was made by removal of a 1.5 inch square of the above mentioned zone from a chromatogram of a known quantity of urine extract. The shredded paper was tamped into a 10-ml serological pipette and elution, performed with 10 ml of absolute methanol, was allowed to proceed at a rate of approximately 0.5 ml per minute. Ultraviolet absorption of eluates was determined on a recording spectrophotometer and concentration computed by comparison with spectra obtained from chromatograms of known amounts of the metabolite model. For investigation of possible glucuronide formation, urine samples were buffered at pH 7 and 2 mg/ml of beta-glucuronidase (100,000 units) was added. After a 24-hour incubation period at 37.5°C, samples were acidified and extracted.

Acetohexamide and metabolite model (Fig. 1) were prepared by Drs. F. J. Marshall and M. V. Sigal.

*Results.* Chloroform extracts from urine of 3 dogs, 2 rabbits, 6 rats, and 4 human subjects that had received oral acetohexamide produced a spot on chromatograms with an $R_f$ 0.56-0.58 in contrast to $R_f$ 0.62-0.64 of acetohexamide. During water elution of the spot white crystals appeared in the eluant. Recrystallization yielded a compound that gave an ultraviolet absorption maximum at 227 m$\mu$ contrasted with an acetohexamide maximum at 246 m$\mu$. Since phenyl alkyl ketones are known to undergo biological reduction to the corresponding alcohols(6), 1-(*p*-$\alpha$-hydroxyethylbenzenesulfonyl)-3-cyclohexylurea was used as a model. A mixed melting point of the metabolite and the model gave no depression. A chromatogram showed a spot of the same mobility as the metabolite. Ultraviolet and infrared absorption spectra as well as the x-ray powder diffraction pattern were identical with the recrystallized metabolite from dog and man.

TABLE I. Twenty-Four-Hour Urinary Excretion of 1-(*p*-α-hydroxyethylbenzenesulfonyl)-3-cyclohexylurea Following Oral Administration of Acetohexamide.

| Species | No. | Oral dose acetohexamide | 24 hr metabolite excretion, % of dose |
|---|---|---|---|
| Dog | 1 | 50 mg/kg | 59 |
| Rabbit | 1 | 50 " | 53 |
| Rat | 6 | 50 " | 18 |
| Man | 2 | 2 g | 49 |

Also, the rat and rabbit metabolites were identified as being the alcoholic derivative on the basis of chromatography and absorption spectra.

Some dog and human chromatograms showed a very slight spot at $R_f$ 0.62, indicating presence of acetohexamide. Eluates of this area from chromatograms of all 4 species failed to produce an absorption maximum at 246 mμ so that excretion of unchanged drug was not further substantiated.

After oral administration of acetohexamide an estimation of the 24-hour urinary recovery of the alcoholic metabolite was made in a dog, a rabbit, 2 patients, and the pooled sample from 6 rats (Table I). Forty-eight hour recoveries for the 2 diabetic patients were 63 and 50%. Metabolite recoveries from 2 other diabetic patients, who received daily doses of 1 g acetohexamide, ranged from 20 to 59%.

Synthetically prepared metabolite was administered to dog and man. In 2 female patients 24-hour urine recoveries were 41% and 61% for doses of 300 mg and 1 g, respectively. Recovery in a dog was 60% of a 50 mg/kg dose. Urine from one of the patients was incubated with beta glucuronidase for 24 hours. No increased recovery of metabolite was noted.

*Discussion.* Tolbutamide [1-(*p*-methylbenzenesulfonyl)-3-butylurea], acetohexamide [1-(*p*-acetylbenzenesulfonyl)-3-cyclohexylurea], and chlorpropamide [1-(*p*-chlorobenzenesulfonyl)-3-propylurea] possess para-substituted phenyl groups. Comparison of the metabolism of these drugs points up a marked influence of the phenyl substituent. In man, tolbutamide (*p*-methyl) is oxidized to a paracarboxylic derivative(1), whereas acetohexamide (*p*-acetyl) is reduced to a para-hydroxyethyl derivative, and chlorpropamide (*p*-chloro) is excreted unchanged or hydrolyzed to *p*-chlorobenzenesulfonamide (5).

Other species have exhibited different metabolic treatment of tolbutamide and chlorpropamide. Mohnike *et al.*(2,3) found that the dog, unlike man, did not oxidize tolbutamide but hydrolyzed it to *p*-methylbenzenesulfonamide or *p*-methylbenzenesulfonylurea. A prior publication from this laboratory(5) showed that the dog excreted chlorpropamide and *p*-chlorobenzenesulfonamide as did man, but in addition excreted 35 to 40% of the dose as *p*-chlorobenzenesulfonylurea. The rabbit was shown to excrete more than 80% of chlorpropamide unchanged with no other detectable metabolites.

Acetohexamide was found to be metabolized alike by dog, rabbit, rat, and man. Recovery of the metabolite from the rat was markedly lower than from the other species, but it was not accompanied by additional metabolites. Since total excretion of drug was not determined, low urinary excretion may be due to poor gastrointestinal absorption and/or biliary excretion of drug or metabolite in this species.

*Summary.* Metabolism of acetohexamide 1-(*p*-acetylbenzenesulfonyl)-3-cyclohexylurea was investigated in dogs, rabbits, rats, and man. Trace amounts of unchanged drug were excreted by dogs and man. All species excreted the reduced form 1-(*p*-α-hydroxyethylbenzenesulfonyl)-3-cyclohexylurea. In a dog, a rabbit and 2 human subjects, 49 to 59% was recovered as metabolite in the urine, but only 18% in the pooled urine of 6 rats. Chromatograms of rat urine revealed no other metabolites.

The authors are indebted to Messrs. D. O. Woolf and L. H. Howard for infrared and ultraviolet absorption data, and to Miss Ann VanCamp for x-ray diffraction patterns.

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Received May 4, 1961. P.S.E.B.M., 1961, v107.

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## Oxidative Effects of 1,1,3-Tricyano-2-Amino-1-Propene. (26695)

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In 1948 it was reported that a solution of malononitrile increased nucleic acid concentration of large nerve cells of the central nervous system of rabbits(1). Since other investigators were unable to reproduce this finding, a series of spectral analyses of fresh and aged solutions of the chemical was undertaken, indicating that active material was probably a decomposition product which formed on standing(2). Isolation and characterization(3) of 1,1,3-tricyano-2-amino-1-propene (U-9189) from similar aged solutions was reported to give the "nucleic acid effect" mentioned above and to uncouple oxidative phosphorylation *in vitro*(4). The compound was found to have antithyroid activity in humans and inhibit thyroxine formation in rats(5) as well as cause pyrexia in rats.* Because this suggested alteration in gross metabolism of treated animals, it was decided to quantitate effects on oxidation rates of various $C^{14}$-labeled compounds in rats.

*Materials and methods.* Adult male albino rats of Upjohn colony (Sprague-Dawley ancestry), weighing 175-180 g and fasted 18 hr (overnight), were used for respiratory studies. Fasted young male rats weighing 60-70 g were used for age comparison studies. Intraperitoneal doses of 50 mg/kg of U-9189 or 15 mg/kg of 2,4-dinitrophenol† (DNP) used in these studies produced slight fevers of 1 to 1.5°F for 1-3 hr in intact and thyroidectomized rats. Doses of U-9189 and DNP were made fresh daily in deionized water or 1/10 N sodium bicarbonate respectively; 1 ml volumes were injected. Some radioactive compounds were injected intraperitoneally as 0.5 ml aqueous solutions of $C^{14}$-2-acetate, sodium ($3.3 \times 10^6$ cpm, 0.45 mg), $C^{14}$-2-glycine ($1 \times 10^6$ cpm, 0.17 mg), $C^{14}$-glucose (uniformly labeled, $1 \times 10^6$ cpm, 0.19 mg), and $C^{14}$-1-DL-leucine ($8.8 \times 10^5$ cpm, 0.27 mg). $C^{14}$-tripalmitin (carboxyl labeled) was incorporated into an emulsion similar to Lipomul‡ and 0.6 ml ($2.4 \times 10^5$ cpm, 0.43 mg of $C^{14}$-1-tripalmitin) injected into a femoral vein.

In a typical experiment, rats were injected with either U-9189 or DNP and ½ hr later with the $C^{14}$ compound and placed in glass metabolic units from which the respired $CO_2$ was collected. Exhaled $C^{14}O_2$ was counted as $BaC^{14}O_3$ by a procedure previously reported (6). In one series of rats, total respiratory $CO_2$ was evaluated by $BaCO_3$ collection, after which thyroid glands were removed. After 10 days of recovery, respiratory $CO_2$ was measured again and rats with 24-30% depression in $CO_2$ output were considered thyroidectomized and used in isotope experiments (Table IV).

*Results and discussion. Oxidation rates by intact adult rats.* Results (Table I) show that, ½ hr after injection of radioactivity, U-9189 increased oxidation rates of all $C^{14}$-labeled compounds except glucose. Compared to their respective controls, peak stimulation of acetate oxidation was 83% at ½ hr, tripalmitin 33% at 1 hr, DL-leucine 61% at 1 hr, and glycine 110% at ½ hr. In view of the fact that U-9189 caused an increased output of total $CO_2$ of about 34% during 5 hr

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* Seay, P. H., McNeil Labs., Philadelphia, Pa., Unpublished data.

† Eastman Organic Chemicals, Rochester, N. Y.

‡ Upjohn trademark for intravenous cottonseed oil emulsion.

TABLE I. Survey of Effects of U-9189 on Cumulative Oxidation of Various Labeled Compounds by Fasting Rats.[*]

| Group | | % of inj. $C^{14}$ oxidized to $C^{14}O_2$ at: ½ hr | 1 hr | 2 hr | 3 hr | 5 hr |
|---|---|---|---|---|---|---|
| $C^{14}$-2-Acetate | Control (5)[†] | 14.0 ± 1.6[§] | 29.4 ± 2.7 | 42.6 ± 3.1 | 48.4 ± 2.4 | 55.3 ± 2.1 |
| | U-9189[‡] (5) | 25.6 ± 1.5 | 44.8 ± 2.4 | 52.3 ± 2.2 | 56.1 ± 3.2 | 65.2 ± 5.1 |
| $C^{14}$-1-Tripalmitin | Control (6) | 6.9 ± .4 | 19.3 ± .9 | 43.6 ± 3.3 | 57.1 ± 4.0 | 72.9 ± 4.6 |
| | U-9189 (6) | 9.0 ± .9 | 24.7 ± 1.9 | 51.6 ± 2.9 | 69.0 ± 5.9 | 80.6 ± 4.2 |
| $C^{14}$-1-Leucine | Control (4) | 5.5 ± .4 | 11.3 ± 1.1 | 18.6 ± .4 | 23.7 ± 1.2 | 27.3 ± 1.0 |
| | U-9189 (4) | 8.5 ± 2.6 | 18.2 ± 2.6 | 26.6 ± 2.7 | 30.2 ± 2.2 | 32.0 ± 1.9 |
| $C^{14}$-2-Glycine | Control (5) | 1.1 ± .2 | 2.5 ± .6 | 4.9 ± .8 | 6.6 ± .9 | 9.9 ± 1.1 |
| | U-9189 (5) | 2.3 ± .1 | 5.1 ± .2 | 8.5 ± .6 | 10.8 ± 1.3 | 17.0 ± 2.5 |
| $C^{14}$-UL-Glucose | Control (4) | 6.3 ± .5 | 18.6 ± 1.0 | 24.0 ± 1.5 | 37.9 ± 1.4 | 45.7 ± 1.7 |
| | U-9189 (4) | 5.7 ± .8 | 21.4 ± 1.6 | 27.7 ± 3.5 | 35.7 ± 4.0 | 45.2 ± 3.5 |

[*] All rats given U-9189 showed an increase of 34 ± 7% in total $CO_2$ exhaled by 5th hr.
[†] No. of rats used to obtain group mean.
[‡] 1,1,3-tricyano-2-amino-1-propene.
[§] Stand. error of mean.

of measurement (Table I footnote), specific activity changes (Table II) are more relevant in evaluating comparative effect of U-9189 on oxidative metabolism. For acetate, leucine, and glycine, increased $C^{14}$ to $C^{12}$ ratio was marked, suggesting increased utilization in excess of that produced by increased metabolic rate. In the case of glycine this was particularly sustained, while with tripalmitin there was a smaller but significant increase lasting less than an hour. Specific oxidation of glucose carbon was definitely less compared to controls, therefore other carbon sources of energy were utilized in greater proportion in presence of the drug.

*Oxidative effects in young and adult rats.* In young rats, 50 mg/kg of U-9189 or 15 mg/kg of DNP had little or no effect on oxidation of acetate (Table III). However, compared to adult rats, young control rats oxidized acetate considerably faster, a rate which apparently could not be further increased by administration of drugs.

TABLE II. Effect of U-9189 on Specific Activity of Total Expired $CO_2$ from Rats Given Various $C^{14}$ Labeled Compounds.[*]

| $C^{14}$-compound oxidized | Specific activity (% of control) at: ½ hr | 1 hr | 2 hr | 3 hr | 5 hr |
|---|---|---|---|---|---|
| $C^{14}$-2-Acetate | 164 ± 10 | 117 ± 3 | 0[†] | 0 | 0 |
| $C^{14}$-1-Tripalmitin | 112 ± 2 | 0 | 0 | 0 | 0 |
| $C^{14}$-1-Leucine | 131 ± 6 | 125 ± 4 | 106 ± 1 | 0 | 0 |
| $C^{14}$-2-Glycine | 184 ± 14 | 153 ± 9 | 128 ± 5 | 128 ± 6 | 128 ± 5 |
| $C^{14}$-UL-Glucose | 74 ± 4 | 83 ± 3 | 84 ± 3 | 85 ± 3 | 88 ± 2 |

[*] Data calculated from $BaCO_3$ collected from experiments recorded in Table I.
[†] No change from controls.

TABLE III. Effect of Age on Cumulative Oxidation of $C^{14}$-2-Acetate by Rats Given Either U-9189 or DNP.[*]

| Group | % of $C^{14}$ oxidized to $CO_2$ at: ½ hr | 1 hr | 2 hr | 3 hr | 5 hr |
|---|---|---|---|---|---|
| Young: Controls (6) | 22.2 ± 2.0 | 42.1 ± 2.2 | 55.5 ± 3.0 | 57.2 ± 3.0 | 62.6 ± 3.4 |
| U-9189 (6) | 26.9 ± 1.7 | 48.7 ± 1.0 | 61.3 ± 1.5 | 64.1 ± 1.7 | 65.7 ± 1.7 |
| DNP (4) | 21.6 ± 3.3 | 41.8 ± 4.2 | 59.4 ± 1.1 | 60.8 ± 1.9 | 67.1 ± 1.2 |
| Adult: Controls (6) | 11.6 ± 1.6 | 26.7 ± 5.4 | 44.9 ± 6.7 | 51.9 ± 4.5 | 58.6 ± 5.3 |
| U-9189 (6) | 25.6 ± 1.5 | 44.8 ± 2.4 | 52.3 ± 2.2 | 56.1 ± 3.2 | 65.2 ± 5.1 |
| DNP (4) | 22.0 ± 1.9 | 44.6 ± 6.9 | 61.4 ± 5.2 | 65.2 ± 6.5 | 73.1 ± 7.0 |

[*] 50 mg/kg I.P. U-9189 or 15 mg/kg DNP increased $C^{14}$ specific activity as well as total exhaled $CO_2$ only in adult rats.

TABLE IV. Oxidation of $C^{14}$-1-Leucine by Fasted Thyroidectomized Rats.*

| | Injected $C^{14}$ oxidized to $C^{14}O_2$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ½ hr | | 1 hr | | 2 hr | | 3 hr | | 5 hr | |
| Group | % O‡ | SA§ | % O | SA | % O | SA | % O | SA | % O | SA |
| Control (6) | 3.5 ±.5 | | 13.7 ± 1.2 | | 20.2 ± 1.9 | | 25.5 ± 2.0 | | 28.7 ± 2.4 | |
| U-9189 (6)† | 3.8 ±.4 | 108 ±1 | 16.6 ± 1.3 | 109 ±2 | 25.2 ± 1.5 | 0 | 30.0 ± 2.4 | 0 | 41.5 ± 3.5 | 0 |

* Effect of U-9189 was tested 2 wk after thyroidectomy in rats specially chosen (see *Methods*). Control values were obtained on these same rats 5 days previous to inj. of U-9189.

† U-9189 treatment increased body temperature and total $CO_2$ output 40% by the 5th hr.

‡ Cumulative % of $C^{14}$ dose oxidized.

§ Specific activity (% of controls), 0 indicates no change from controls.

*Oxidation by thyroidectomized rats.* U-9189 increased the metabolic rate of fasted thyroidectomized rats also. Both cumulative $C^{14}O_2$ from $C^{14}$-leucine, as well as total $CO_2$, increased about 40% by the fifth hour (Table IV and footnote, Table IV). It is interesting to note that, in spite of a similar increase in $CO_2$, intact rats showed a greater increase in $C^{14}O_2$ specific activity during the first hour, *e.g.*, 25-31% (Table II), while operated rats showed only a slight increase of 8-9% (Table IV). Absence of thyroid gland therefore altered overall oxidative pattern. Presence of thyroid hormone (permissive action) for maximal response to reproductive hormones has been reviewed(7), but the nature of relationship between U-9189 and thyroid hormone suggested here awaits further experimentation.

Since the drug increased metabolic rate of thyroidectomized rats, it is unlikely that this action resulted from induced thyroid hormone secretion in intact rats. The mechanism whereby U-9189 increased metabolism *in vivo* was probably due to uncoupling of oxidative phosphorylation mentioned previously. However, U-9189 does affect the thyroid gland *per se* in that it can depress thyroid function as mentioned earlier. In view of this, it is interesting that a similar combined effect has been reported for salicylates. They have been shown to uncouple oxidative phosphorylation by a manner different from that reported for thyroxine(8) and to depress thyroid function(9). Salicylates have also been shown to release thyroxine from serum proteins(10) and it is possible that decreased $C^{14}$ specific activity response obtained from thyroidectomized rats given U-9189 was related to lack of bound thyroid hormone which may have been released had it been present.

*Summary.* Administration of 1,1,3-tricyano-2-amino-1-propene (U-9189) increased total $CO_2$ output and oxidation of $C^{14}$-labeled acetate, tripalmitin, DL-leucine, and glycine in intact fasting rats. U-9189 also increased $C^{14}$ specific activity of exhaled $CO_2$ suggesting increased utilization in excess of increased metabolic rate. Total oxidation of $C^{14}$-glucose was not influenced; resultant specific activity was therefore decreased. Although in thyroidectomized rats U-9189 produced a similar increase in total $CO_2$ expired and oxidation of $C^{14}$-DL-leucine, specific activity of total $CO_2$ increased only slightly compared to intact rats given U-9189. Total "oxidative effect" of U-9189 appeared to be lacking in the absence of thyroid gland.

The authors are indebted to Dr. Floyd S. Eberts, Jr. for a sample of U-9189.

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Received May 8, 1961. P.S.E.B.M., 1961, v107.

# A Comparison of Canine Distemper and Measles Viruses.* (26696)

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A relationship between canine distemper (CDV) and measles (MV) viruses has been suggested on the basis of similar histopathology(1,2) and antigenic cross reactions(3, 4,5). Since evidence of a relationship has to date been primarily biological and the serological findings have been subject to controversy(6), it seemed pertinent to compare physical properties of these viruses. The filterability, and sensitivity to irradiation, heat and ether of CDV and MV have been studied.

*Materials and methods. Virus stocks.* Canine distemper virus kindly supplied by Dr. G. Rockborn(7) was grown in dog kidney tissue culture with a medium composed of Earle's salt solution plus 0.5% lactalbumin hydrolysate and 2% calf serum. Stock virus titers up to $10^4$ per 0.1 ml were obtained. The Edmonston strain of measles virus was grown in Hep-2 cultures with Eagle's medium plus 10% calf serum; titers of $10^5$ per 0.1 ml were obtained. *Titrations.* CDV titrations were carried out in tubes at half log intervals and checked for cytopathogenic effect at 7-14 days. The measles titrations were assayed in African monkey (*Erythrocebus patas* or *Cercopithecus aethiops*) kidney cultures by the plaque method. *Gamma irradiation.* Both viruses were subjected to gamma radiation from the Cobalt-60 source of the Yale University Dept. of Biophysics. This unit delivered 270,000 roentgens per hour. *UV irradiation.* The viruses were exposed to UV radiation from a Westinghouse G 30T8 Sterilamp which gave 1590 ergs/sec/cm$^2$ as measured by a SM-600 meter (Westinghouse). *Gradocol membranes* were obtained from the Wright-Fleming Institute, London.

*Results.* The identity of the virus stocks was confirmed by titration against human sera collected before and after measles, and against sera from dogs before and after distemper. None of the "pre" specimens neutralized either virus. Post CDV specimens neutralized the CDV only, while post-measles specimens neutralized MV to high titer and CDV at low titer. Typical antibody titers are given in Table I.

TABLE I. Neutralization of Virus Strains.

| | Canine sera | | Human sera | |
|---|---|---|---|---|
| | Pre-CD | 3 yr post-CD | Pre-measles | 2 mo post-measles |
| CDV | <2 | 45 | <2 | 6 |
| MV | <2 | <2 | <2 | 128 |

Filtration experiments were carried out to obtain an estimate of the size of the CDV. The results are given in Fig. 1. On some occasions infectious virus passed membranes with a rated average pore diameter (APD) as low as 180 m$\mu$, but never smaller. In several tests it was held back by membranes with APD values as high as 250 m$\mu$, but it passed every membrane with larger diameters. On the basis of a ratio of 0.64 between virus and pore diameter(8) these data would indicate a size for CDV between 115 and 160 m$\mu$, a range similar to that previously reported for measles virus(9).

Inactivation curves of CDV and MV by gamma radiation are presented in Fig. 2.† The slopes of the curves for the 2 viruses were indistinguishable in both wet and frozen preparations. The difference between the 2 viruses is always less than a log unit and hence within the range of error to be expected from the endpoint method. Unfrozen (0°C) preparations of both viruses showed a more rapid rate of inactivation than frozen (–70°C) specimens. The diameter of the effective targets of CDV and MV based on gamma sensitivity in the wet preparations was 42 m$\mu$. When calculated on the basis

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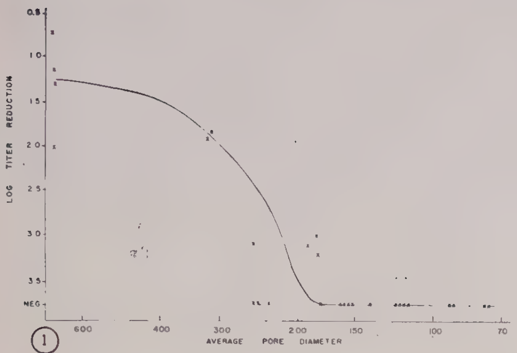
[*] This investigation was supported by a grant from Nat. Inst. Health, U.S.P.H.S.

[†] The data for measles inactivation by gamma rays at 0°C and by UV were obtained by Mr. Paul Ackerman, Yale Univ. School of Med.



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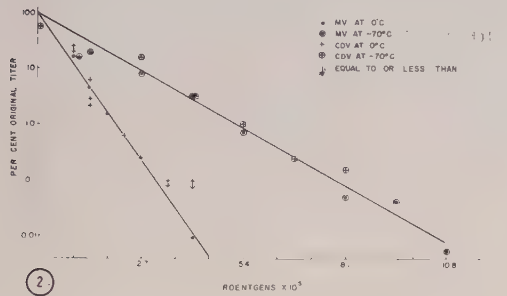

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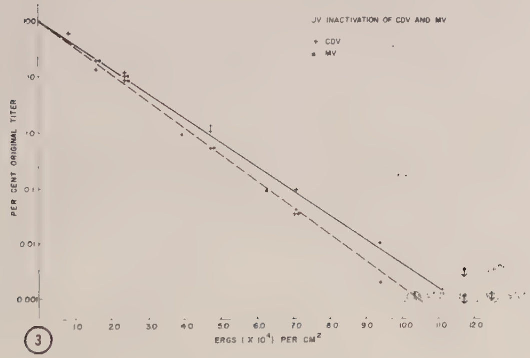

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FIG. 1. Filtration of canine distemper virus through collodion membranes. Relations between avg pore diameter and reduction of titer in filtrates.

FIG. 2. Inactivation of canine distemper and measles viruses by irradiation from a cobalt-60 source at two temperatures.

FIG. 3. Inactivation of canine distemper and measles viruses by irradiation with ultraviolet light at 22°C.

of the frozen preparations, a figure of 35 mμ was obtained for the diameters of both viruses.

Data on sensitivity of the 2 viruses to UV are shown in Fig. 3. This non-ionizing form of radiation gave similar rates of inactivation for CDV and MV. Both curves were exponential over a 5 log range and titer of both viruses was reduced about 1.0 log unit per 22,000 ergs/cm$^2$.

Tests for thermal sensitivity were carried out with undiluted virus from tissue culture fluids. Preparations of each virus were sealed in ampules and immersed in water baths held at 37°C and 56°C. Ampules were removed at several intervals and titrated. Titer of both viruses fell 1.2 to 1.4 log units in 1 hour at 37°C and both were reduced more than 3.7 log units in 8 hours at 37°C or ½ hour at 56°C. Thus, as has been previously reported with MV(10), CDV was relatively labile at elevated temperatures.

Both CDV and MV were sensitive to ether. Preparations of both viruses shaken with an equal volume of ether and allowed to stand at 4°C overnight were completely inactivated, whereas controls without ether retained full titer.

*Discussion.* Biological and serological properties have frequently been used to group various viruses simply because they have been the properties most readily investigated. However, physical and chemical criteria may reflect more fundamental characteristics of viruses and hence offer some advantages. Since there has been controversy regarding the serological relationship between CDV and MV, the paucity of data from other lines of investigation has been particularly noticeable. The present study has shown CDV and MV to be similar with respect to size, and sensitivity to irradiation, to heat, and to ether; hence, our observations support the hypothesis that these viruses are members of a common group.

The filtration data put both viruses in the same general size range as herpes virus, varicella, salivary gland and rabies viruses. MV and CDV differ from herpes virus in their greater sensitivity to UV and ionizing radiation(11) and from rabies in their sensitivity to ether. Physical and chemical data on salivary gland and varicella viruses are insufficient to provide a basis for comparison.

The rate of inactivation by gamma radiation found in these studies at 0°C was only slightly less than that reported to occur with measles in serum free media at 25°C by Musser and Underwood(12). However, the viruses were considerably more resistant to gamma rays when in frozen media. This latter effect was in contrast to results obtained with poliovirus(9) but similar to vaccinia results obtained by J. F. McCrea Freezing presumably suppresses indirect effects of irradiation either through restricting mobility of radicals formed in the medium, or by reducing transfer of charges from non-essential to essential portions of the virus particle. Regardless of the mechanism, the effect of freezing provides another point of similarity between the viruses under consideration.

*Summary.* Comparisons of certain physical properties of canine distemper (CDV) and measles (MV) viruses have yielded data compatible with the hypothesis that they belong to the same group. Ultrafiltration of CDV indicated a particle size between 115 and 160 mμ, a value similar to that previously reported for measles. Both viruses were inactivated by ultraviolet light at a rate of 4.5 log units per erg/cm$^2$ × $10^5$. Irradiation with $10^5$ roentgens of gamma particles reduced the titer 0.4 log unit at −70° and 1.0 log unit at 0°C for both CDV and MV.

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Received May 8, 1961. P.S.E.B.M., 1961, v107.

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## Disappearance Rate of Normal Bactericidins in Irradiated Mice.* (26697)

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In an earlier publication(1) we reported that normal serum bactericidal activity for a strain of *Escherichia coli* was lost within 9-12 hours after total body exposure of CF-1 mice to a midlethal dose of X-rays and remained absent for about 10 days. The disappearance of bactericidins could not be associated with development of an inhibitor demonstrable *in vitro* or with *in vivo* absorption by enteric microorganisms through the damaged intestinal mucosa.

The rapidity of the loss of bactericidins indicated that their production was promptly interrupted by irradiation and that they must have a very short biological half-life. Recent findings suggested that a block in the synthesis of bactericidins develops after X-ray exposure(2). It is the purpose of this communication to show that normal bactericidins for *E. coli* disappear very quickly after passive transfer in irradiated mice.

*Materials and methods.* CF-1 mice were exposed to 500 or 700 r total body X-radiation‡ and were used on the following day when no serum bactericidal activity was demonstrable. They were injected intravenously *via* a tail vein with pooled mouse sera of known bactericidin titer and exsangui-

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* This work was supported in part under U. S. Atomic Energy Commission Contract.

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† Operated by University of Chicago for U. S. Atomic Energy Commission.

‡ The authors wish to thank Mr. James Bland for irradiating the animals.

nated at intervals thereafter. Bactericidal tests were performed with sera from individual mice. Briefly, the test consisted of mixing serially diluted mouse serum, rabbit complement and a suspension of *E. coli*. After incubation at 37°C, the number of microorganisms present in each tube was determined either by plating aliquots on eosin-methylene blue agar and making colony counts or by adding 5 volumes of nutrient broth and continuing incubation until the complement control became turbid. At that time, the presence or absence of turbidity in all other tubes was recorded. These readings closely paralleled the results obtained by plate counts. The reciprocal of the highest dilution of serum showing bactericidal activity was recorded as the titer. Details regarding animals, irradiation factors, bleeding and assay of bactericidins have been reported(1). In one series of experiments, preinjection plasma samples were obtained from each mouse by a slight modification of the orbital bleeding technic described by Riley(3).

Immune sera for sheep cells were obtained from CF-1 mice given 1 ml of a 1% suspension of washed sheep erythrocytes intraperitoneally 12-13 days earlier. For hemagglutination tests, equal volumes (0.1 ml) of serial 2-fold serum dilutions and 1% washed sheep cells were mixed and allowed to stand at room temperature for 30 minutes. The tubes were then centrifuged for 5 minutes at 1000 rpm and gently shaken. The reciprocal of the highest dilution of serum showing agglutination distinctly visible to the naked eye was recorded as the titer.

*Experimental. Disappearance of normal bactericidins following a single intravenous injection.* One day postirradiation, mice were injected intravenously with 0.25 ml of mouse serum having a bactericidin titer of 128. Control mice were given 0.25 ml of negative serum (titer<2). Table I lists the titers of the recipients' sera at various intervals after injection. Bactericidins were not demonstrable in sera of uninjected mice or of those given negative serum. Bactericidins were present in the circulating blood for a short time after injection of positive serum and were lost so rapidly as to be undetectable 20 minutes later.

*Disappearance of normal bactericidins following a second intravenous injection.* To rule out the possibility that the rapid disappearance of bactericidins might be due to equilibration between intravascular and extravascular fluids, a second intravenous injection of bactericidal mouse serum was given 30 minutes after the first. The results of 5 separate experiments are summarized in Table II. After one injection, passively transferred bactericidins always disappeared within 30 minutes. After 2 injections, passively transferred bactericidins had disappeared by 30 minutes in half of the animals, and by 60 minutes in all those tested. It is interesting to note that the mean titers of sera from

TABLE I. Disappearance of Passively Transferred Bactericidins after a Single Intravenous Injection.*

| Serum inj. .25 ml | Bleeding time, min. after inj. | No. mice tested | No. of mice with titer of: | | | | | | | Geometric mean titer |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | <2 | 2 | 4 | 8 | 16 | 32 | 64 | |
| Positive (titer 128) | 1 | 20 | | 2 | | 2 | 9 | 4 | 3 | 17.1 |
| | 10 | 6 | | | | 2 | 3 | 1 | | 14.2 |
| | 15 | 6 | 3 | | | 1 | 2 | | | |
| | 20 | 6 | 6 | | | | | | | |
| | 30 | 10 | 9 | | 1 | | | | | |
| | 45 | 3 | 3 | | | | | | | |
| | 60 | 8 | 8 | | | | | | | |
| | 120 | 3 | 3 | | | | | | | |
| Negative (titer <2) | 1 | 11 | 11 | | | | | | | |
| | 10 | 6 | 6 | | | | | | | |
| | 30 | 3 | 3 | | | | | | | |
| | 60 | 3 | 3 | | | | | | | |
| None | | 18 | 18 | | | | | | | |

* Mice were exposed to 500 r 24 hr before inj.

TABLE II. Disappearance of Passively Transferred Bactericidins after 2 Intravenous Injections 30 Minutes Apart.*

| No. of injections† | Bleeding time, min. after inj. | No. mice tested | No. of mice with titer of: <4 | 4 | 8 | 16 | 32 | 64 | Geometric mean titer |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 17 | | | 3 | 12 | 2 | | 15.3 |
| | 30 | 9 | 9 | | | | | | |
| 2 | 1 | 12 | | 1 | 3 | 4 | 3 | 1 | 16.0 |
| | 30 | 13 | 7 | 1 | 2 | 2 | 1 | | |
| | 60 | 12 | 11 | 1 | | | | | |

* Mice were exposed to 700 r 24 hr before inj. All mice had preinjection titers of <4.
† 0.25 ml mouse serum (titer 128).

mice sacrificed immediately after injection were essentially the same for both groups. In this series of experiments, a bleeding was obtained from each mouse before the first injection of serum. All initial plasma samples were devoid of bactericidal activity.

*Disappearance of immune hemagglutinins after passive transfer.* Dixon *et al.*(4) reported that homologous gamma globulins (antibodies) had a half-life of 1.9 days in mice. This value differs considerably from that we obtained for normal bactericidins. Consequently, disappearance rates of normal bactericidins and immune hemagglutinins were compared under identical experimental conditions. A mouse antiserum for sheep cells which was also bactericidal was given intravenously to irradiated mice. This serum had a hemagglutinin titer of 1024 and a bactericidin titer of 128. The recipients were sacrificed at intervals after injection and their sera were tested for the presence of both *E. coli* bactericidins and sheep cell agglutinins. The results are presented in Table III. In this experiment, as in those described above, a loss of passively transferred bactericidins corresponding to about 3 serum dilutions occurred within 30 minutes. In contrast, 5 days were required for a comparable decline in sheep cell agglutinins.

*Discussion.* Taliaferro and Talmage(5) have pointed out that the half-life of antibodies should be calculated from disappearance rates after passive transfer, not from rates of antibody decline after immunization. Values based on the latter are generally too high as they reflect rates of synthesis as well as of decay. The same considerations apply if the half-life of naturally occurring antibodies is calculated from disappearance rates after whole body irradiation(6).

To determine the rate of metabolic decay of normal bactericidins for *E. coli,* passive transfer experiments were carried out in irradiated mice whose sera were not bactericidal. Presumably, bactericidins were not syn-

TABLE III. Disappearance of Passively Transferred Bactericidins and Sheep Cell Agglutinins.*

| Bleeding time after inj.† | No. mice tested | Bactericidins<br>No. mice with titer of: <4 | 4 | 8 | 16 | 32 | Hemagglutinins<br>No. mice with titer of: 8 | 16 | 32 | 64 | 128 | 256 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 min. | 5 | | 1 | 1 | 3 | | | | | | 3 | 2 |
| 30 ” | 6 | 6 | | | | | | | | | 6 | |
| 6 hr | 6 | 6 | | | | | | | | | 6 | |
| 1 day | 6 | 6 | | | | | | | | 6 | | |
| 2 days | 4 | 4 | | | | | | | 1 | 3 | | |
| 3 ” | 4 | 4 | | | | | | 1 | 1 | 2 | | |
| 4 ” | 4 | | | | | | | | 4 | | | |
| 5 ” | 3 | | | | | | | 1 | 2 | | | |
| 6 ” | 4 | | | | | | 1 | 3 | | | | |
| 7 ” | 4 | | | | | | | 4 | | | | |

* Mice were exposed to 500 r 24 hr before inj. Uninjected mice had bactericidin and hemagglutinin titers of <4.
† 0.20 ml mouse serum (bactericidin titer 128; hemagglutinin titer 1024).

thesized during the period of observation, therefore their half-life could be estimated from the time of their disappearance after intravenous administration.

The data presented indicate that the half-disappearance time of homologous bactericidins after passive transfer in irradiated mice was a few minutes. Equilibration between intravascular and extravascular fluids accounted for the distribution of bactericidins immediately after intravenous injection, but not for their complete absence 20 minutes later. Estimating the plasma volume of a 25 g mouse to be 1.5-2 ml, 0.25 ml of intravenously injected serum would be diluted 6-8-fold. Thus, a serum of titer 128 should produce titers of approximately 16 in the recipients. Mice sacrificed 1 minute after injection of such a serum had mean bactericidin titers of 17.1 and 15.3 (Tables I and II).

If the loss of bactericidins during the first 30 minutes after intravenous administration had been solely the result of equilibration, it should have been possible to prolong their survival considerably by reinjecting a bactericidal serum at the end of this interval. However, after the second injection, bactericidins disappeared almost as rapidly as after the first.

Data obtained with the sheep cell immune serum provide additional evidence that the rapid disappearance of bactericidins is not due to equilibration. The initial dilution factors were the same for bactericidins and hemagglutinins. Thereafter, the half-disappearance times were a few minutes for the former and approximately 2 days for the latter.

Our findings with hemagglutinins are in accord with the results of Dixon, *et al.*(4) who reported that the half-life of homologous antibodies in mice was 1.9 days, and with those of Smith and co-workers(7) who studied passively transferred antisheep hemolysins.

Our data are consistent with the premise that the rapid disappearance of passively administered bactericidins in irradiated mice is due to metabolic decay. They do not, however, provide information on whether such decay is more rapid in irradiated than in normal mice. Hollingsworth(8) found that irradiation did not alter the rate of disappearance of passively transferred antibodies, but it is not known whether this holds true also for other serum proteins.

We reported earlier(1) that normal serum bactericidins for *E. coli* were lost 9-12 hours after total body exposure of CF-1 mice to X-rays. The data presented here show that such bactericidins, when injected into irradiated mice, were lost in less than 1 hour. In other words, the disappearance of passively transferred bactericidins in previously irradiated mice was much more rapid than was the disappearance of normally circulating bactericidins from mice following their exposure to total body irradiation. Similar observations on hemolysins in rabbits were made by Taliaferro and Talmage. They found the mean half-disappearance time of rabbit antisheep hemolysins (large molecules), as determined by passive transfer, to be 2.8 days(5), but that of natural hemolysins in irradiated rabbits to be 7 days(6). If a comparison may be made between hemolysins in rabbits and bactericidins in mice, the loss of normal serum bactericidal activity 9-12 hours after total body exposure of CF-1 mice to 600 r(1) may be explained as follows: production of bactericidins is impaired shortly after irradiation, resulting in an overall rate of decline more gradual than in absence of bactericidin synthesis. A few hours post-irradiation, bactericidin production ceases altogether for approximately 10 days.

*Summary.* X-irradiated CF-1 mice lacking normal serum bactericidal activity for *Escherichia coli* were injected intravenously with highly bactericidal mouse serum. Passively transferred bactericidins were lost so rapidly from the circulating blood as to be undetectable 20-30 minutes later. Following 2 intravenous injections 30 minutes apart, bactericidins disappeared almost as quickly as after a single injection. The disappearance rates of normal bactericidins and immune hemagglutinins were compared under identical experimental conditions by injecting a mouse antiserum to sheep cells which was also bactericidal. The half-disappear-

ance time of sheep cell agglutinins in mice was approximately 2 days, that of normal bactericidins, only a few minutes.

The authors are indebted to Dr. William H. Taliaferro for his interest in this work, and for many valuable suggestions.

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Received May 12, 1961. P.S.E.B.M., 1961, v107.

## Lipid Metabolism in Cultured Cells. II. Cholesterol Uptake from Serum of Normal and Atherosclerotic Human Adults.*† (26698)

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It has been shown that mammalian cells in culture take up considerable amounts of cholesterol from the serum used in the growth medium(1). It was found that the cholesterol content of cells was dependent both upon the type of cell cultured, and upon the type of serum used. Cholesterol uptake by cells was only indirectly related to cholesterol level in the medium, and appeared to be related more directly to the ratio of cholesterol to other unidentified components of the serum(2). The primary process in development of atherosclerotic lesions appears to be related to cellular deposition of cholesterol in the intima of the arterial walls(3). It was of interest therefore to determine if any differences could be found in deposition of cholesterol in cells cultured *in vitro* upon serum taken from normal subjects as compared with serum from atherosclerotic individuals.

*Materials and methods.* Blood samples taken from normal and atherosclerotic subjects following overnight fasting were allowed to clot for 24 hours at 4° before centrifuging and collection of serum. Details of age, sex and clinical diagnosis of experimental subjects are given in Table I. Sterile conditions were maintained during collection, and serum samples were stored frozen at –40° until use. The strain of cells used in the studies reported here, the MB III strain of mouse lymphoblasts, was grown in roller tube cultures by methods described previously(1). Serum samples were deactivated by heating at 55° for 30 minutes before use. This heating procedure destroyed a toxic factor associated with most fresh serum samples. It did not affect the cholesterol content of cells grown on non-toxic serum.

Stock cultures of MB III cells in logarithmic growth phase, were harvested and washed and resuspended in Gey's balanced saline solution. Cell populations were determined by counting in a hemocytometer chamber, and aliquots of the suspension containing approximately 1 million cells were dispensed in duplicate into roller tubes containing the experimental serum samples. Gey's balanced salt solution was then added

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* This investigation was supported by research grants from Nat. Heart Inst., and from Nat. Cancer Inst., U.S.P.H.S.

† We are grateful to Drs. George S. Mirick and Roger Akel (Baltimore City Hospital) and Dr. Martin Singewald (Johns Hopkins Hospital) for assistance in obtaining serum samples.

TABLE I. Relationship between Serum Cholesterol, Age, and Cholesterol Content of Cells Grown on Serum in Normal and Atherosclerotic Human Subjects.

| Normal adults | | | | Atherosclerotics | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cholesterol | | | | | Cholesterol | |
| Sex | Age | In serum, mg % | In cells, % | Sex | Age | Diagnosis | In serum, mg % | In cells, % |
| ♂ | 24 | 154 | 3.3 | ♂ | 77 | M.I. | 173 | 3.9 |
| ♂ | 26 | 170 | 3.2 | ♂ | 61 | ” | 420 | 7.9 |
| ♂ | 29 | 164 | 3.0 | ♀ | 62 | ” | 199 | 3.2 |
| ♂ | 29 | 213 | 3.2 | ♂ | 55 | C | 273 | 4.3 |
| ♂ | 29 | 142 | 3.3 | ♂ | 52 | A | 220 | 3.8 |
| ♂ | 30 | 128 | 2.8 | ♂ | 53 | A | 186 | 4.0 |
| ♂ | 30 | 146 | 3.0 | ♂ | 75 | A | 248 | 3.6 |
| ♂ | 31 | — | 3.1 | ♀ | 60 | CVA | 218 | 3.4 |
| ♂ | 31 | 206 | 3.7 | ♀ | 56 | ” | 189 | 4.3 |
| ♂ | 31 | 207 | 4.3 | ♀ | 76 | ” | 232 | 4.8 |
| ♀ | 32 | 189 | 3.3 | ♀ | 82 | CBS | 222 | 4.6 |
| ♂ | 37 | 225 | 3.6 | ♀ | 54 | CVA | 292 | 3.3 |
| ♂ | 40 | — | 4.0 | ♂ | 80 | ” | 214 | 3.8 |
| ♀ | 41 | 195 | 3.6 | ♀ | 75 | CBS | 226 | 4.4 |
| ♂ | 41 | 162 | 3.0 | ♀ | 68 | CVA | 227 | 4.4 |
| ♀ | 45 | 219 | 3.7 | ♂ | 74 | ” | 306 | 3.3 |
| ♀ | 48 | 257 | 4.8 | ♂ | 71 | ” | 226 | 4.3 |
| ♀ | 57 | 324 | 4.6 | ♂ | 73 | ” | 166 | 4.3 |
| ♂ | 58 | 197 | 4.3 | ♂ | 72 | ” | 240 | 4.2 |
| ♀ | 58 | 280 | 4.2 | ♂ | 57 | M.I. | 181 | 3.3 |
| | | | | ♀ | 80 | CBS | 226 | 3.5 |
| Mean | 37.35 | 198.8 | 3.60 | ♀ | 63 | CVA | 187 | 4.4 |
| S.D. | ±10.5 | ±49.2 | ±.575 | ♂ | 75 | ” | 242 | 4.9 |
| | | | | ♂ | 88 | CBS | 185 | 4.4 |
| | | | | ♂ | 91 | ASHD | 167 | 3.8 |
| | | | | ♂ | 83 | CVA | 186 | 3.7 |
| | | | | ♀ | 73 | CBS | 256 | 3.5 |
| | | | | ♂ | 82 | ” | 240 | 3.3 |
| | | | | ♀ | 78 | ” | 298 | 4.0 |
| | | | | Mean | 70.6 | | 229.1 | 4.09 |
| | | | | S.D. | ±34.2 | | ±53 | ±.86 |

Stand. error of difference in cell cholesterol = .205.
Difference of mean = .49. $P < .01$.

*Key to letter diagnosis:* M.I., myocardial infarct; C, coronary occlusion; CVA, cerebro vascular accident; CBS, chronic brain syndrome; ASHD, arterio-sclerotic heart disease; A, angina pectoris.

so that final concentration of serum in the medium was 50%. Cells were grown for 4 days during which time the average population increase was 4-fold. The cells were harvested by centrifugation followed by washing with 2 successive portions of balanced saline to remove traces of medium. Cell lipids were extracted by refluxing with 5 ml of a 1:1 mixture of ethanol-ether for 30 minutes, and the extract was evaporated to dryness in a stream of nitrogen. The residue was then extracted twice with boiling petroleum ether (B.Pt. 30-60°) and cholesterol in the extract was determined by the method of Brown(4). The extracted cells were air dried at 110° for 1 hour and dry weight was determined by the colorimetric method of Bailey and Nejad(5). The cholesterol content of experimental serum samples was determined by using a similar double solvent extraction procedure.

In a second series of experiments a group of 6 rabbits were rendered atherosclerotic by feeding a diet containing 2% cholesterol in 5% lard for 3 months. A control group of 6 rabbits were maintained on a cholesterol-free diet. At the end of the 3 month period the animals were sacrificed and blood was obtained under sterile conditions by heart puncture. The cholesterol-fed group all had well developed plaques in the thoracic aorta, whereas the control group had none. Cells were grown on the serum obtained from both groups of animals and cholesterol content of

TABLE II. Cholesterol Content of Cells Grown on Normal and Atherosclerotic Rabbit Serum.

| | No. in group | Mean serum cholesterol, mg % | Mean cell cholesterol, % |
|---|---|---|---|
| Control rabbits | 6* | 85.8 ± 27.1 | 7.80 ± 2.2 |
| Atherosclerotic rabbits | 6 | 2148 ±498 | 22.25 ± 2.89 |

* Results taken from ref. (2).

the harvested cells and the serum was determined as described above.

*Results and discussion.* The results of the experiments with cells grown on human serum samples are given in Table I. The mean serum cholesterol levels in the atherosclerotic group were significantly higher (13.6%) than those of the normal group. Cells grown on the atherosclerotic serum had a significantly higher cholesterol level than those grown on normal serum (4.09% *vs* 3.60%) and the mean elevation was again 13.6% suggesting a direct correlation between serum and cell cholesterol (see below). Examination of the group of normal sera shows a highly significant correlation between age of the donor and cell cholesterol level (correlation coefficient r = 0.73); however there was also a high correlation between serum cholesterol level and age of the donor, (r = 0.85) and also between serum cholesterol level and cell cholesterol (r = 0.86). In the atherosclerotic group there was a significant correlation between serum cholesterol and cell cholesterol (r = 0.5) but no significant correlation between age and cell cholesterol (r = 0.04) or age and serum cholesterol (r = 0.06). These results were analyzed by covariant analysis and the following conclusions were reached:—The increase in cell cholesterol with age of the donor in the normal group is entirely associated with the increase in serum cholesterol with age and the related correlation between serum cholesterol level and cell cholesterol. (r for cell cholesterol with age *independently* of serum cholesterol = 0; r for cell cholesterol with serum cholesterol *independently* of age = 0.67). The absence of significant correlation of serum cholesterol with age in the atherosclerotic group is to be expected in view of the high average age of this group (Table I). It has been shown that most of the increase in serum cholesterol occurs between 0 and 60 years of age(6).

The results for normal and atherosclerotic rabbits are given in Table II. The rabbits on the cholesterol diet showed a 20-25 fold increase in serum cholesterol over the 3-month period. Cells grown on the atherosclerotic rabbit serum had about 3 times the cholesterol content of those grown on normal rabbit serum. All rabbits in the cholesterol fed group developed 4+ plaques when graded according to the nomenclature of Wang(7). The interesting fact that the species from which the serum is derived is important in determining cell cholesterol levels, is apparent in these results. Normal rabbit serum has a lower cholesterol than normal human serum, yet grows cells with a higher cholesterol content. This apparent anomaly may be related to the ease with which this animal is rendered atherosclerotic by dietary means alone.

There is therefore no evidence in the experiments reported here for normal and atherosclerotic humans which suggests that factors other than the increase in serum cholesterol which occurs with ageing, are responsible for either the higher cholesterol content of cells grown on the atherosclerotic sera, or the increase in cell cholesterol with age of the donor in normal subjects. In view of the high average age of the atherosclerotic group (71 years) it may be questionable to consider this group as truly abnormal, since atherosclerosis is a fairly normal accompaniment of aging in this range. A more informative group of patients for experiments such as these might be atherosclerotics of lower age, so that comparative studies with young normals could be made with elimination of the age factor. The difficulty of obtaining sufficient numbers of patients in the latter category has prevented such a study from being carried out.

*Summary.* 1. The cholesterol content of cells grown on serum samples taken from normal and atherosclerotic humans and rabbits was determined. 2. Cells grown on atherosclerotic sera had significantly higher

cholesterol levels than those grown on normal sera. 3. In the group of normal human sera there was significant correlation between (a) age of the donor and cholesterol content of cells, (b) serum cholesterol level and cholesterol content of cells and (c) age of the donor and serum cholesterol level. 4. In the atherosclerotic human sera there was significant correlation between serum and cell cholesterol but none with age of the donor. 5. It appears from these results that in an older group of human atherosclerotics at least, there is no evidence that the higher cholesterol content of cells grown on the serum is due to factors other than the increase in serum cholesterol which occurs with aging.

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Received May 15, 1961. P.S.E.B.M., 1961, v107.

# Increase of Plasma Lysozyme Activity Following Injections of Typhoid Vaccine.* (26699)

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Kerby has shown that white cells incubated *in vitro* with endotoxin from Gram-negative bacteria leak lysozyme into the fluid in which they are suspended(1). The studies reported here were undertaken to see whether injections of endotoxin into rabbits resulting in leukopenia were followed by an increase in plasma lysozyme activity.

*Materials and methods.* The lysozyme activity of rabbits' plasma was measured after intravenous injection of endotoxin or Newcastle disease virus (NDV). Blood was obtained from unanesthetized rabbits by repeated cardiac puncture using a syringe containing 0.3 ml of 5% sodium ethylene diamine tetraacetic acid/10 ml of blood.† Plasma was obtained free of cells by 2 centrifugations at 1000 rpm for 20 minutes at 4°C, and was stored at 4° until the assay of lysozyme activity was performed. Most of the assays were done within 8 hours after collection of blood, and none was delayed more than 24 hours. Pyrogen free glassware and syringes were used in preparation of plasma.

Lysozyme activity was measured by the rate at which plasma samples cleared turbid suspensions of *Micrococcus lysodeikticus*. Irradiated cell walls of *M. lysodeikticus* ("lysozyme substrate"—Difco Corp., Detroit, Mich.) were diluted in phosphate buffer pH 6.2 ("lysozyme buffer"—Difco) to a concentration of 0.5 mg/ml. Plasma was diluted in buffer 1/20, 1/40, or 1/80. Standard solutions containing 0.8 and 1.0 $\mu$g/ml of crystalline egg white lysozyme (Difco) were prepared in buffer.

Four ml of substrate and 4 ml of diluted plasma or standard solution were mixed in a cuvette and optical density was determined at 5 minute intervals in a Bausch and Lomb Spectronic 20 at 540 m$\mu$ wavelength. All tests were caried out at 32°C and the reactants were allowed to reach this temperature before mixing. The lysozyme concentration of plasma was determined by comparing the

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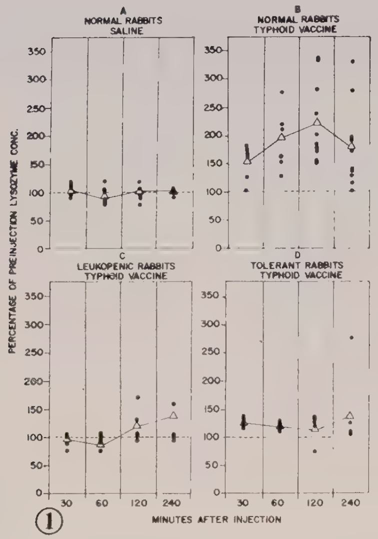
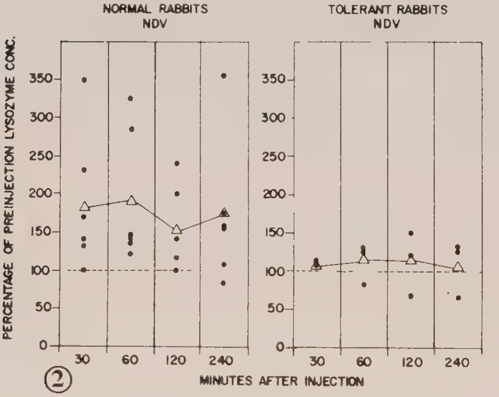
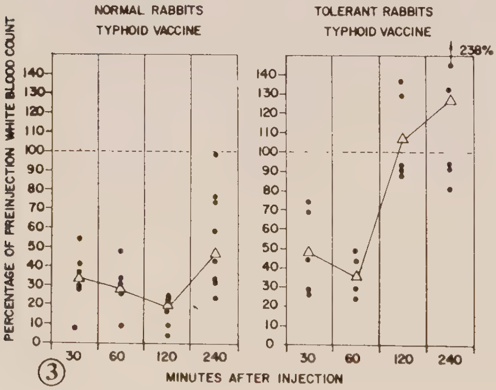
* This study was supported by a contract with U. S. Army Chemical Corps, Fort Detrick, Frederick, Md.

† Plasma of citrated blood had almost no detectible lysozyme activity.







● = individual observations
△ = avg of observations

FIG. 1. Plasma lysozyme concentrations at intervals after intrav. inj. of saline or typhoid vaccine into rabbits.

FIG. 2. Lysozyme concentrations after intrav. inj. of Newcastle disease virus into rabbits.

rate of lysis observed with known amounts of purified egg white lysozyme.

White blood cells were counted in the blood removed from the heart for lysozyme assay.

*Results. Injection of endotoxin into normal rabbits.* The resting lysozyme concentration in 27 rabbits varied from 4 to 36 $\mu$g/ml with an average of 11.1 $\mu$g/ml ± 8.0 (S.D.). Because of the variation the data are recorded as percentage of pre-injection lysozyme concentrations.

Intravenous injection of $4 \times 10^8$ heat killed *Salmonella typhosa* in 1.0 ml of saline into 13 rabbits was followed without exception by leukopenia (Fig. 3) and by an increase in plasma concentration of lysozyme (Fig. 1B). The maximum increase was noted 2 hours after injection, and by 4 hours the concentration of lysozyme had declined slightly. The increases in lysozyme concentration in animals injected with typhoid vaccine roughly paralleled the degree of leukopenia observed. Three rabbits injected with 1.0 $\mu$g of a purified endotoxin from *Salmonella abortus equi* (supplied by Dr. Otto Westphal) showed similar increases in lysozyme concentration suggesting that endotoxin was the agent in the typhoid vaccine responsible for the rise in concentration. The rabbits injected with pyrogen free saline had little change in lysozyme concentration (Fig. 1A) and no change in white blood cell count.

*Leukopenic rabbits.* Because of the studies of Kerby(1) and the correlation of the increased lysozyme concentrations with development of leukopenia it seemed likely that the white blood cells were a major source of the lysozyme released into the plasma. Five rabbits were made leukopenic by a single intravenous injection of 6.0 mg of nitrogen mustard ("Mustargen"—Merck, Sharp & Dohme). When the concentration of circulating granulocytes in the blood was less than 1000/mm$^3$ (usually about 48 hours after injection of nitrogen mustard) the animals were injected intravenously with typhoid vaccine in the same dose used for

FIG. 3. White blood counts in blood of normal and tolerant rabbits following inj. of typhoid vaccine.

normal rabbits, and lysozyme concentrations were measured. The pre-injection lysozyme concentration in the plasma of these rabbits was in the normal range. At 30 and 60 minutes after injection of typhoid vaccine there was *no increase* in lysozyme concentration. At 120 and 240 minutes there was only slight increase to an average of 120% of the preinjection value (Fig. 1C). These results suggest that the lysozyme released into the plasma comes from white cells, damaged in some way by typhoid vaccine.

*Tolerant rabbits.* Rabbits repeatedly injected with endotoxin gradually become refractory to many of the actions of endotoxin, and it seemed possible that lysozyme release might also be decreased in tolerant rabbits. Five rabbits made tolerant by daily intravenous injections of $4 \times 10^8$ heat-killed *S. typhosa* for 2 weeks were studied for the effect of a single injection of typhoid vaccine on lysozyme concentration in the plasma. Three days elapsed between the last injection of the 2-week course and the study of changes in lysozyme concentration brought about by another intravenous injection. There were small increases in lysozyme concentration in the plasma of tolerant rabbits but much less than in non-tolerant rabbits (Fig. 1D). An incidental finding was the short duration of leukopenia in tolerant as compared to non-tolerant rabbits (Fig. 3).

*Injection of Newcastle disease virus.* It seemed of interest to see whether lysozyme concentration changed in rabbits following injection of other agents. Preliminary studies on rabbits injected intravenously with 512 hemagglutinating units of NDV showed an increase in plasma lysozyme activity as great, in some rabbits, as the increase in rabbits injected with endotoxin (Fig. 2). The peak of the rise was 60 minutes after injection and was not accompanied by a marked leukopenia. Animals made tolerant to the pyrogenic action of NDV by an injection of 512 hemagglutinating units 24 hours before challenge responded with a smaller increase in lysozyme concentration (Fig. 2).

*Discussion.* These data indicate that plasma lysozyme concentration can be increased by injections of endotoxin from Gram-negative bacteria. It seems likely that the source of the enzyme is damaged granulocytes in view of the finding by Kerby(1) of a release of lysozyme from granulocytes incubated *in vitro* with endotoxin. The lack of increase in lysozyme concentration in granulocytopenic rabbits after endotoxin injection adds further support to this view.

Atkins and Wood(2) have shown that after injection of endotoxin into rabbits an "endogenous pyrogen" appears in the serum that can cause fever when injected into other rabbits. This substance was not found when endotoxin was injected into granulocytopenic rabbits and they hypothesized that white cells were the source of "endogenous pyrogen." The experiments reported here give further indication that white cells in animals injected with endotoxin are damaged and leak some of their contents.

Tolerant rabbits do not show an increase in lysozyme activity after injection of endotoxin. This may result merely from the rapid removal of endotoxin from the blood of tolerant rabbits making it inaccessible to white blood cells. Another possibility put forward by Cluff(3) is that white cells from tolerant animals are relatively resistant to damage by endotoxin. It is interesting that the endotoxin-induced leukopenia in tolerant rabbits is of shorter duration than that in non-tolerant rabbits even though the immediate leukopenia is just as marked. If white cells of tolerant rabbits are not damaged by endotoxin they may be only sequestered outside the circulating blood (accounting for the immediate leukopenia) but not destroyed. The undamaged leukocytes then may return to the blood stream. On the other hand cells from non-tolerant rabbits may be sequestered and destroyed resulting in a prolonged leukopenia.

*Summary.* 1. Intravenous injection of typhoid vaccine, purified endotoxin and Newcastle disease virus into rabbits results in elevation of plasma lysozyme concentrations. 2. This elevation of lysozyme activity after injection of typhoid vaccine is not seen in leukopenic rabbits or in rabbits made tolerant to endotoxin. 3. Leukopenia in non-tolerant animals injected with endotoxin is prompt and is still present at 4 hours after the injection. In tolerant rabbits the leukopenia is apparent immediately, but by 2 hours after injection the white cell count has returned to normal levels.

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Received May 15, 1961. P.S.E.B.M., 1961, v107.

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# Various Types of Calcinosis Induced by Egg Albumen or Yolk Following Sensitization with Dihydrotachysterol (DHT).* (26700)

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In rats pretreated with dihydrotachysterol (DHT), it is possible to induce intense local calcinosis anywhere on the skin surface by topical application of mechanical trauma(1) or subcutaneous injection of $FeCl^3$(2). The lesions so induced are somewhat reminiscent of the calcifying form of scleroderma (the "sclérodermie calcaire" of French dermatologists). Recently we found that subcutaneous injections of either egg white or egg yolk are likewise highly effective in eliciting cutaneous calcinosis, following DHT sensitization, although neither of these substances produces any significant local damage (*e.g.*, inflammation, hemorrhage or necrosis). It appears that the active principles of the egg —like $FeCl_3$—act somewhat like mordants, in that they prepare tissues for subsequent uptake of calcium salts. Even when administered intraperitoneally or intravenously, both egg white and egg yolk (unlike $FeCl_3$) are well tolerated by rats; hence, they enabled us to study the influence of systemically applied vital mordants for calcium upon DHT-sensitized animals.

*Material and methods.* Fifty female Holtzman rats, with a mean initial body weight of 97 g (range: 92-105 g), were subdivided into 5 equal groups. All animals were given 1 mg of DHT ("Calcamin," Wander), in 0.5 ml of corn oil, by stomach tube, once on the first day. The rats of *Group I* acted as controls and received no other treatment, while those of the remaining groups received solutions of either egg white or egg yolk, 24 hours after the DHT. The solutions were prepared by mixing the albumen or yolk of the eggs of domestic fowl with an equal amount of distilled water. The resulting thick fluids tend to form stringy precipitates upon standing and do not pass readily through ordinary filter paper; hence, they were filtered first through cotton and then through tissue paper ("Kleenex"®). The individual dose was invariably 5 ml, egg white being given intraperitoneally to *Group II* and intravenously to *Group III*, while egg yolk was administered intraperitoneally to *Group IV* and intravenously to *Group V*. The animals were kept on "Purina Fox Chow" and tap water throughout the experiments and all survivors were killed with chloroform after 4 days. At autopsy their organs were inspected with a stereoscopic loupe, representative specimens of various tissues being fixed in alcohol-formol for the subsequent histochemical demonstration of calcium with von Kóssa's silver nitrate technic.

*Results.* Five animals of the group receiving egg yolk intravenously died during the period of observation, but all the other rats supported these injections well, except that egg white produced the usual anaphylactoid reaction during the first few hours after its administration. Even this response was mild, since it is well known that large amounts of

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* Supported by grants from Nat. Institutes of Health, U.S.P.H.S., Geigy Pharmaceuticals and the Gustavus and Louise Pfeiffer Research Foundation.

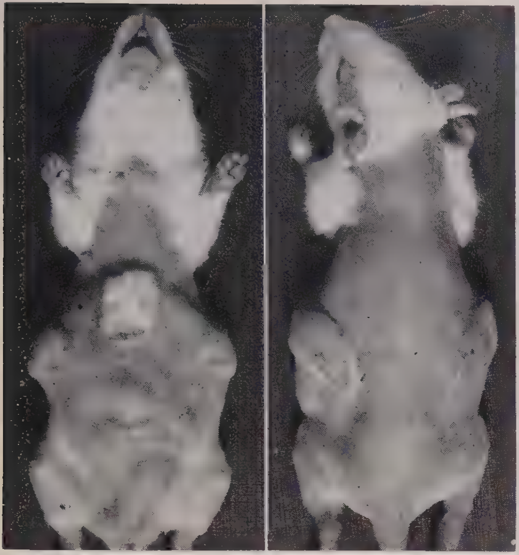

FIG. 1. The 3 typical calcified skin plaques in a rat treated with DHT (p.o.) + albumen (i.p.).

egg white are less effective in this respect than smaller doses(3).

At the dose used, *DHT alone* (Group I) produced only occasional traces of calcification in gastric mucosa, coronary arteries or kidneys.

About 48 hours after *intraperitoneal injection of egg white* (Group II), in all animals, 3 well-circumscribed cutaneous plaques appeared. One patch was situated just above the cartilaginous tip of the xiphoid bone; the other 2, in the costovertebral angles. The lesions were extremely painful to touch and while at first they adhered to the underlying muscle layers, by the fourth day it became possible to move them more or less freely over the subcutaneous tissue (Fig. 1). Histologically, the plaques proved to consist of heavy calcium deposits in the various muscular, fascial and dermal layers between the peritoneum and the epidermis through the entire thickness of the abdominal wall.

The most striking internal lesion was the virtually complete calcification of the pancreatic stroma, which imparted a dense, chalky appearance to the organ. Here, calcium deposition could be demonstrated with particular clarity by fixing the entire loop of duodenum, with the pancreas attached, in alcohol-formol and subsequenlty staining with $AgNO_3$ (Fig. 2A). Histologic examination showed that the calcification is strictly limited to the pancreatic stroma and consists of extremly fine $AgNO_3$-positive granules surrounded by inflammatory changes and intense sclerosis. There was no trace of stainable calcium in the excretory parenchyma, the ducts or the Langerhans islets (Fig. 2B). On the other hand, heavy calcification was also regularly found in the middle layer of the gastric mucosa, the duodenum (especially around Brunner's glands), the retroperitoneal fat and the diaphragm. The kidney exhibited only traces of corticomedullary calcinosis, while the omentum, liver, spleen and adrenal remained unaffected. In the thorax, heavy deposits of calcium were seen on the pleura, the pericardium and throughout the thickness of the thoracic wall, especially around the sternum. The thymus and pulmonary parenchyma showed little if any calcification, but the lymph nodes around the thymus were heavily calcified. Calcium deposition in the coronary arteries was not definitely in excess of the trace amounts produced by this dose of DHT in control animals.

*Intravenous injection of egg white* (Group III) caused an increase in the intensity of calcification in the coronary arteries. In addition, heavy calcium deposits were often seen in the auricular appendages and the sub-

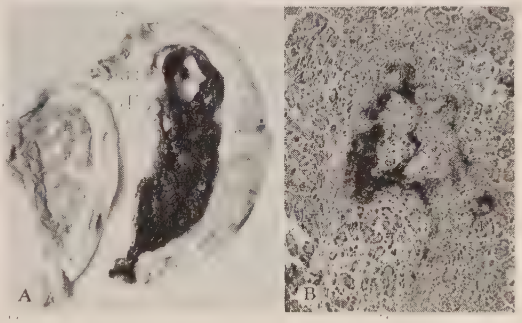

FIG. 2. Pancreatic calcification in a rat treated with DHT (p.o.) + albumen (i.p.). *A*, left: Duodenal loop with pancreas of a rat that received DHT alone, and comparable specimen of an animal treated with DHT + albumen. Both specimens were defatted and stained with silver nitrate to demonstrate calcification. *B:* Histologic aspect of calcification in pancreas of rat shown in Fig. 1. The calcium deposits are extremely fine and are surrounded by a great excess of inflamed connective tissue. (v. Kóssa × 60.)

epicardial portions of the ventricular musculature; these regions never showed calcification in the controls treated with DHT alone.

Following *intraperitoneal injection of egg yolk* (Group IV), there was no calcification of the skin or abdominal wall, although the omentum (which was not affected by intraperitoneal egg white) showed intense calcification. Furthermore, the Kupffer cells and some collagen fibers that surround the hepatic sinusoids likewise exhibited severe calcification. The thoracic organs were only mildly affected, except for the perithymic lymph nodes, which receive lymph-borne substances from the peritoneal cavity. This dissimilarity in the distribution of the calcium deposits produced by albumen and yolk, respectively, is presumably due to differences in their direct and lymphatic spreading. Control experiments in similarly DHT-sensitized animals revealed that, if introduced directly into the subcutaneous tissue, both albumen and yolk induce approximately equal degrees of cutaneous calcinosis.

The most striking effect of *intravenous injection of egg yolk* (Group V) was an intense calcification of the spleen, making the organ whitish, hard and brittle. There was also considerable calcification of the Kupffer cells and of the isolated connective tissue fibers around the hepatic sinusoids. The hepatic parenchyma, blood vessels and biliary ducts remained unaffected. This rather selective distribution of calcification is presumably due to the fact that the lipid-containing particles of egg yolk are preferentially taken up by the splenic and hepatic reticuloendothelium.

*Discussion.* Following sensitization with DHT, it is possible to produce, by intraperitoneal injection of albumen or yolk, rather selective calcification affecting certain tissues. It is difficult to explain the pathogenesis of the calcification caused by intraperitoneal injection of albumen in all layers of the abdominal wall and particularly in the skin over the xiphoid cartilage and the costovertebral angles. However, the abdominal wall of the rat is especially thin in these locations, which may account for the ready outward spreading of the egg white. The calcification of the thoracic organs may be ascribed to the circumstance that the principal lymphatic channels of the peritoneum ascend along the thoracic wall and, hence, presumably carry egg white upward.

Distribution of the calcification is quite different when egg white is injected intravenously. Yet other types of calcinosis—each with a constant and typical distribution pattern—are produced by intraperitoneal or intravenous injection of egg yolk, which possesses a particular affinity for the reticuloendothelial system. Numerous control experiments (not detailed here) have shown that neither albumen nor yolk causes any trace of calcification in otherwise untreated rats. Furthermore, hypercalcemia produced by other means (parathyroid hormone, sodium acetylsulfathiazole, nephrectomy combined with oral administration of calcium acetate) can similarly sensitize the rat for production of ectopic calcification by "vital mordants". On the other hand, the effect of albumen and yolk can be duplicated by various injectable iron preparations (ferric oxide saccharate, iron dextran). Hence, this type of "vital mordanting" is not limited to the particular pathogenic agents used in the present experiments.

Special importance is attached to the intense inflammation and sclerosis that accompanies and follows calcification in this type of lesion. In many experiments performed in the manner described here, we have seen that, if rats are allowed to survive for 2 or 3 weeks, the fine, dust-like calcium deposits (especially in pancreas, abdominal wall and thoracic organs) are rapidly absorbed and dense sclerotic tissue takes their place. Finely dispersed calcium-dust cannot be detected without the use of special stains; yet it can lead to inflammation and sclerosis. The frequent association of derangements in calcium metabolism and collagen disease (*e.g.*, the hypercalcemia of sarcoidosis, the ectopic calcification in scleroderma) suggests that further studies along these lines may help in elucidation of various systemic diseases affecting connective tissue.

*Summary.* In rats previously sensitized by a single oral dose of dihydrotachysterol

(DHT), massive and rather selective calcification of the pancreas is induced by subsequent intraperitoneal administration of egg white. Intravenous injection of egg yolk elicits a calcinosis primarily affecting the reticuloendothelial cells of the liver and the spleen. Yet, other types of calcinosis are induced by intravenous injection of egg white or the intraperitoneal administration of egg yolk. It is assumed that albumen and yolk act as "vital mordants," preparing the tissues of suitably sensitized animals for the subsequent uptake of calcium, distribution of the lesions being dependent upon the special distribution patterns of the mordants themselves.

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Received May 15, 1961. P.S.E.B.M., 1961, v107.

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## Metabolism of Human Amnion Cell Cultures Infected with Poliovirus. II. Utilization of Glucose, Pyruvate and Ribose in Virus Infected Cells.

(26701)

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Human amnion cell cultures(1) proved to be a suitable system for investigation of effects induced by poliovirus on the metabolism of the host. It was shown(2) that infected cells utilized glucose by the glycolytic pathway. Levy and Baron(3) showed poliovirus to stimulate glycolysis of monkey kidney cells, and Matzelt(4) found increased activity of the glycolytic enzymes in a similar cell-virus system. This paper is a report of an investigation of the effect of glucose, ribose and pyruvate on virus multiplication. In addition, observations on the synthesis of lactic acid and ribose in infected and uninfected cells are also reported.

*Materials and methods. Preparation of cell cultures.* Primary human amnion-cell cultures were prepared as previously described(2). Monolayers were formed within 5-7 days.

*Virus.* A stock of the virus was prepared by inoculating amnion monolayers in milk bottles with the $MEF_1$ strain of poliovirus type II. After incubation for 72 hours at 37°C the medium was centrifuged, and the supernate containing the virus preserved at $-20$°C (infectivity titer $10^{7.0}$/ml). Virus titers were determined by inoculating tubes of amnion cells with 10-fold dilutions of the virus suspensions, using 4 tubes per dilution, and following the cytopathic changes microscopically for 7 days. The $TCID_{50}$ of the virus preparations was determined by the method of Reed and Muench(5).

*Infection of cell-cultures.* The cell cultures were washed with PBS, inoculated with the virus to give a multiplicity of infection of 5-10, and incubated for 1 hour at 37°C; the unadsorbed virus was removed by washing the cell with PBS. Eagle's medium, prepared with glucose, ribose or pyruvate or without any carbohydrate, was added to the cultures, which were reincubated at 37°C.

*Chemical determinations.* Aliquots of infected and uninfected cells or of cell hydrolysates were used for quantitative determinations of glucose, pyruvate, lactic acid and ribose. Glucose was determined by the Somogyi-Nelson photometric method(6), lactic acid by the method of Barker and Summerson(7), ribose (found in the medium and in the cells) by the method of Drury(8) and pyruvate by reaction with dinitrophenyl hydrazin reagent(9). For determinations of cell-synthesized-ribose, the cells were washed with 3 changes of PBS, released from the

TABLE I. Lactic Acid Production in Uninfected and Poliovirus Infected Human Amnion Cell Cultures.

| Hr after reincubation* | Infected cell cultures |  |  | Uninfected cell cultures |  |
|---|---|---|---|---|---|
|  | Eagle's medium† containing: |  |  |  |  |
|  | Glucose | Ribose | No carbohydrate | Glucose | Ribose |
|  | Lactic acid formed ($\mu g/10^6$ cells) |  |  |  |  |
| 0 | 85.5‡ | 35.0 | 56.5 | 60.0 | 20.0 |
| 5 | 169.0 | 35.0 | 70.0 | 102.0 | 50.0 |
| 18 | 505.0 | 35.0 | 70.0 | 300.0 | 50.0 |

* Cell cultures were incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus, Eagle's medium added, and cells reincubated.

† Eagle's medium was modified to contain glucose, ribose (500 $\mu g/ml$) or no carbohydrate at all.

‡ Results of cumulative lactic acid formation are expressed in $\mu g/10^6$ cells originally present in culture.

glass by combined versene and trypsin treatment(10), counted in hemocytometer, and hydrolyzed with alkali (by heating in a boiling water bath for 1 hour with 0.4 N NaOH).

*Results. Relation between glucose uptake and lactic acid production.* We have previously reported(2) the increased uptake of glucose by poliovirus infected amnion cell cultures. The present report deals with the lactic acid formed by uninfected and infected cultures.

Both uninfected and infected amnion cell cultures yielded lactic acid when glucose was used as substrate. However, when glucose was replaced by ribose, or when no carbohydrate was added, no lactic acid was formed (Table I). The increase of lactic acid formation in infected cells grown on glucose coincided with the period of virus synthesis (between 7-12 hours after addition of the virus (Fig. 1). During this period glucose was taken up very actively by these cells.

In uninfected amnion cells, the molar ration between lactic acid formed and glucose consumed was 1.56, showing that 80% of the glucose was utilized through the glycolytic pathway (Table II). In the infected cells the lactic acid:glucose ratio was 0.9, indicating that only 46% of the glucose was transformed to lactic acid. This lower ratio was obtained despite the fact that in poliovirus infected cells, glucose uptake was twice as high as in uninfected cells (2.93 $\mu M$ and 1.43 $\mu M$ per $10^6$ cells, respectively).

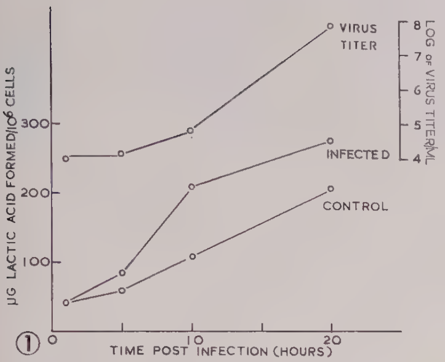
*Effect of poliovirus on pyruvate uptake.* Primary amnion monolayers incubated in Eagle's medium with pyruvate or ribose during an observation period of 36 hours showed the same morphological characteristics as cultures in presence of glucose. Pyruvate consumption, as measured by its disappearance from the medium, amounted to 2-4 $\mu$moles per 24 hours. Cells infected with poliovirus showed diminished uptake of pyruvate, beginning 5-7 hours after infection. However, the cells continued to incorporate pyruvate during the next 20 hours. Virus release from cells incubated with pyruvate was only 1% of that formed in presence of glucose (Table III).

*Effect of poliovirus on ribose uptake.* Poliovirus infected cells incubated in Eagle's

TABLE II. Glucose Consumption and Lactic Acid Formation in Infected and Uninfected Human Amnion Cell Cultures.

| Exp. No. | Infected cells* |  |  | Uninfected cells |  |  |
|---|---|---|---|---|---|---|
|  | Glucose consumed, $\mu M$ | Lactic acid formed, $\mu M$ | Lactic acid / Glucose | Glucose consumed, $\mu M$ | Lactic acid formed, $\mu M$ | Lactic acid / Glucose |
| 1 | 2.0 | 1.8 | .90 | 1.6 | 2.5 | 1.56 |
| 2 | 3.1 | 2.8 | .90 | 1.6 | 2.4 | 1.50 |
| 3 | 3.7 | 3.6 | .97 | 1.1 | 1.8 | 1.63 |

* Cell cultures incubated with poliovirus for 1 hr at 37°C, washed with PBS to remove unadsorbed virus, Eagle's medium added and cells reincubated.



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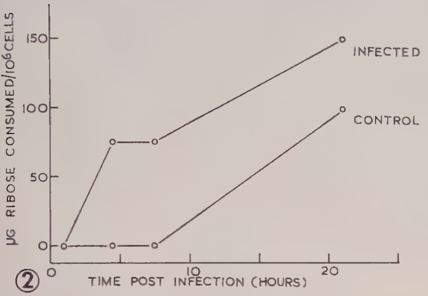

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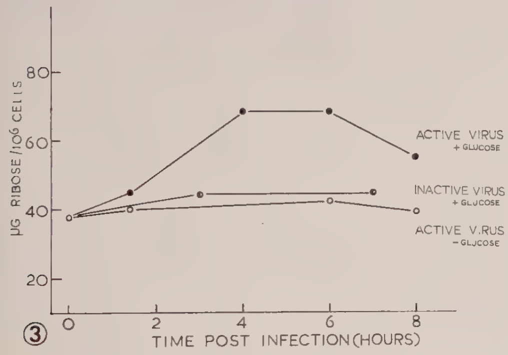

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FIG. 1. Correlation between lactic acid formation and poliovirus production in human amnion cell cultures. Cell cultures incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus, Eagle's medium added and cells reincubated. Amount of virus was determined by inoculating tubes of amnion cells with 10-fold dilutions of the virus. See *Methods.*

FIG. 2. Ribose consumption by poliovirus infected amnion cells. Cell cultures were incubated with poliovirus for 1 hr at 37°C, then washed with PBS to remove unadsorbed virus. Eagle's medium containing ribose was added, and cells were reincubated.

FIG. 3. Increase of intracellular ribose content during poliovirus infection. Amnion cell cultures were incubated with poliovirus in Eagle's medium with or without glucose. At various intervals, cells were washed with PBS, released from the glass by combined versene and trypsin treatment, counted in a hemocytometer, hydrolysed with alkali, and ribose content determined.

TABLE III. Effect of Ribose and Pyruvate on Poliovirus Production by Amnion Cell Cultures.

| Eagle's medium* containing: | Net increase of virus titer,† log/ml | P‡ |
|---|---|---|
| Glucose | 4.0 ± .0 § | |
| Ribose | 2.5 ± .15 | <.01 |
| Pyruvate | 1.88 ± .23 | <.01 |
| No carbohydrate | 2.0 ± .24 | <.01 |

* Cell cultures incubated with poliovirus for 1 hr at 37°C, washed with PBS to remove unadsorbed virus, Eagle's medium added, and cells reincubated. Results obtained after incubation for 20 hr.

† Log virus titer obtained by subtracting the initial virus titer in the culture fluid from the titer of virus reached 24 hr post-infection.

‡ Probability of obtaining a larger value of *t* by chance. Level of significance is $P < 0.05$.

§ Mean ± stand. error (S.E.) of mean. S.E. = $\sqrt{\frac{\Sigma d}{n(n-1)}}$ (Ref. 13).

medium containing ribose (instead of glucose), took up the sugar immediately and to a greater extent than uninfected controls (Fig. 2). However, despite the increased uptake of ribose by infected cells, the amount of virus was no greater than that produced by cells incubated in Eagle's medium without added carbohydrate (Table III). The virus yield was only 1-5% of that obtained from cells incubated in presence of glucose.

*Effect of poliovirus on intracellular ribose synthesis.* Infection with poliovirus stimulated intracellular ribose synthesis in cell cultures containing glucose. Formation of ribose began 2-4 hours after infection and continued for 6 hours, *i.e.*, up to the appearance of cytopathic changes, then the production of intracellular ribose decreased. Uninfected cells, or those incubated with heat inactivated poliovirus showed no change in intracellular ribose content. Ribose synthesis was not observed in infected cells incubated in Eagle's medium lacking glucose (Fig. 3).

*Discussion.* The metabolism of primary human amnion cell cultures is mainly glycolytic, as indicated by the conversion of 80% of the glucose consumed into lactic

●—●, infected cells in a medium containing glucose; ○—○, infected cells in a medium without added carbohydrate; ◒—◒, cells in a glucose containing medium incubated with heat inactivated virus.

acid. No lactic acid was formed by cells in the absence of glucose or in presence of ribose (instead of glucose). Infection of cell cultures with poliovirus stimulated lactic acid formation. Similar findings were made by Levy and Baron in poliovirus infected monkey kidney cell cultures(3). However, determination of the lactic acid formed is not sufficient to evaluate the metabolic pathways of the infected cells. The infected cells not only produced more lactic acid, but consumed much more glucose than uninfected controls. Thus, whereas in uninfected cells the ratio of lactic acid formed to glucose consumed is about 1.6, in infected cells it is only 0.9.

It was previously shown(2) that inhibitors of the oxidative pathways, *i.e.*, cyanide and azide, had no effect on poliovirus formation. We have shown in the present paper that pyruvate utilized aerobically by infected cells did not support virus synthesis. This affords an additional proof that oxidative pathways are not involved in virus formation.

The metabolic shift in the infected cells as shown by the low lactic acid:glucose ratio does not necessarily indicate an increase in oxidative metabolism. Rather, it is assumed that during infection glucose possibly takes part in metabolic processes which do not lead to lactic acid formation.

When ribose was substituted for glucose in the medium it was found to be incorporated by cells immediately after infection. Despite the stimulated ribose uptake, the virus yield was only 1-5% of that formed in presence of glucose, as much as in cells incubated in absence of any added carbohydrate (Table III). The findings that ribose cannot substitute for glucose in poliovirus production may perhaps be explained by not participating in energy yielding processes which lead to lactic acid formation (Table I). Darnell and Eagle(11) also showed that ribose could not support polio-virus replication in HeLa cell cultures. However, no data were represented by them to show whether ribose was utilized by these cells.

Determinations of intracellular ribose in infected cells showed an increase in ribose content during 6 hours and a decrease following the appearance of cytopathic changes in the cells. Ribose was formed only when glucose was present in the medium, whereas none was formed in its absence. The finding of increased intracellular ribose synthesis may be connected with the increased RNA synthesis in poliovirus infected HeLa cells as described by Ackerman(12).

*Summary.* 1. Infected amnion cells take up more glucose and produce more lactic acid than uninfected controls. However, the lactic acid:glucose ratio is 0.9 in infected and 1.6 in control cells. This finding indicates that poliovirus infected amnion cells use glucose through different metabolic channels than uninfected cells. 2. Both infected and uninfected cultures take up ribose from the medium, the infected cells being more active. The ribose consumed cannot supply the proper carbohydrate source for virus formation. Infected, but not uninfected cells, synthesize ribose, if glucose is present in the medium. 3. Both infected and uninfected cells take up pyruvate but its uptake is diminished following infection. Pyruvate cannot replace glucose for virus synthesis.

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Received May 15, 1961. P.S.E.B.M., 1961, v107.

# Cross-Protection Between Venezuelan Equine Encephalomyelitis and Eastern Equine Encephalomyelitis Virus. (26702)

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The viruses of the equine encephalitides have been reported to be related antigenically(1). Evidence of cross-protection among these viruses following immunization with inactivated viruses is lacking(2,3,4). However, immunity induced by injection of live virus appears to be cross-protective among certain of the group A arthropod-borne viruses. Stamm and Kissling(5) reported cross-protection between live eastern and western strains of equine encephalomyelitis virus in sparrows and horses, and Parks and Price (6) showed that guinea pigs injected with the live western strain resisted peripheral challenges with Sindbis virus and vice-versa. Casals(1) reported that 2 injections of live Chikungunya virus resulted in an appreciable resistance in mice to challenge by Semliki Forest virus. More recently it was found (7) that one injection of an attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus, strain 9t, enabled mice and monkeys to resist large lethal doses of the virulent homologous strain given intracerebrally, and that the high immunizing efficiency of the vaccine strain was related to a limited viral multiplication which was found in the absence of clinical illness(8). These findings suggested that cross-protection among group A arthropod-borne viruses should be examined further. In these experiments, mice immunized by either the subcutaneous, intraperitoneal, or respiratory route with 9t were challenged with serial dilutions of a chick embryo seed preparation of eastern equine encephalomyelitis (EEE) virus. In addition, experiments were carried out in which animals that were injected with EEE virus were challenged with a virulent VEE virus strain.

*Materials and methods. Virus strains.* The attenuated VEE virus, 9t, used to immunize mice was a tissue culture seed prepared after its isolation from a chronically infected L-cell culture followed by 9 passages in fresh L cells. This strain represents an additional passage in L cells of the CILV-8 strain which was described elsewhere(7). The virulent parent egg seed, PES, of VEE virus used to challenge immunized animals also has been described(7).

The EEE virus seed, EE-2, was isolated from the brain of a human case, passed 8 times in mouse brain and 3 times in chick embryos. In preparation of the 4th passage egg embryo seed, 0.1 ml of a $10^{-3}$ dilution of EE-2 was inoculated into the allantoic cavity of 10-day eggs. Embryos harvested at 24 hours were prepared as virus seeds in the same manner as for the PES strain(7).

*Strain differences as determined by animal response to injection of virus.* The differences in virulence of PES, 9t and EE-2 viruses were determined as follows: serial 10-fold dilutions of each strain were injected into 5- to 6-kg rabbits intraperitoneally (i.p.), into 200-to 300-g guinea pigs i.p. and into 4-day chicks subcutaneously (s.c.). Animals that withstood doses of $10^6$ $MICLD_{50}$ of virus or less were considered as resistant to that virus; animals which succumbed to $10^2$ $MICLD_{50}$ were considered susceptible.

*Strain differences as determined by serum neutralization tests.* Neutralization tests with anti-9t or anti-PES monkey sera or anti-EE-2 rabbit sera were used to verify differences among virus strains. They were performed in mice according to the method described elsewhere(7). Neutralization tests were also caried out in L-cell(9) cultures that had been grown for 48 hours in Leighton tubes containing medium 199(10) supplemented with 20% horse serum. Fifteen-hundredths (0.15) ml of serial 10-fold dilutions of virus in Bacto heart infusion broth (BHIB) were incubated for 30 minutes at room temperature with 0.15 ml antiserum which was diluted 1:1 in BHIB. The mixture was then added in 0.2 ml amounts to duplicate culture tubes containing 1 ml of

TABLE I. Comparative Resistance of Mice Challenged with a Virulent Strain of VEE Virus (PES) or EEE Virus (EE-2) after Immunization with Attenuated VEE Virus (9t).

| Route of immunization | Challenge virus | Route of challenge | Titer MICLD$_{50}$ (log$_{10}$) of challenge virus in 9t immunized mice | Titer MICLD$_{50}$ (log$_{10}$) of challenge virus in Nonimmunized mice | Protective index* (log 10) |
|---|---|---|---|---|---|
| Intraperitoneal† | PES | Intraperitoneal | $<1.6$ | 8.9 | $>7.3$ |
| | | Intracerebral | 2.8 | 9.2 | 6.4 |
| Respiratory‡ | | Intraperitoneal | $<1.6$ | 8.6 | $>7.0$ |
| Intraperitoneal | EE-2 | Intraperitoneal | 2.2 | 6.4 | 4.2 |
| | | Intracerebral | 7.1 | 8.7 | 1.6 |
| Respiratory | | Intraperitoneal | 3.5 | 6.6 | 3.1 |

* Difference in No. of LD$_{50}$ resisted by immunized and nonimmunized animals.

† Immunization dose was $10^{3.4}$ MICLD$_{50}$. Similar results varying no more than 0.3 log in last column were obtained after subcut. immunization with 9t.

‡ Calculated immunization dose of $10^{3.4}$ MICLD$_{50}$ given by 5-min. exposure to aerosols of 9t.

tissue culture medium. Control tubes were inoculated with mixtures of virus and normal serum, or virus and BHIB.

The tubes were examined microscopically every 24 hours for 6 days for evidence of a cytopathic effect (cpe) due to virus multiplication in the cell monolayer. Virus titers were determined on the basis of the highest virus dilution which produced a cpe in 50% of the tubes and were expressed as number of CPED$_{50}$(11) per ml of virus suspension. The neutralization index was computed as the difference between CPED$_{50}$ titers obtained with normal serum or BHIB and those with antiserum. A neutralization index of less than 10 was considered not significant.

Tests for formation of plaques were carried out according to a method described elsewhere(12) except that neutral red was added in combination with a second agar overlay.

*Virus immunizations.* Twelve- to fourteen-gram mice were vaccinated with $10^{3.4}$ MICLD$_{50}$ of 9t given i.p. or s.c. Vaccinations by the respiratory route were carried out by exposing animals to aerosols of 9t for 5 minutes in a modified Henderson apparatus (13). Mice received maximum aerosol doses of $10^{3.4}$ MICLD$_{50}$, which were calculated on the basis of 100% retention of total number of MICLD$_{50}$ in the volume of air inhaled within a 5-minute exposure period. Rabbits and guinea pigs were immunized against EEE virus by an i.p. injection of $10^8$ and $10^6$ MICLD$_{50}$, respectively of EE-2; the hosts were resistant to these doses.

*Virus challenges.* Challenge doses of virus were administered to immunized animals at 21 days postvaccination. Controls consisted of animals which came from the same group as those that were immunized. They were, therefore, comparable to unimmunized animals with respect to age, weight and length of time in contact with the same laboratory cage facilities. Mice immunized i.p., s.c., or by aerosol exposure were challenged i.p. or i.c., with 10-fold dilutions of EE-2 or PES diluted in BHIB. Challenges with $10^{5.3}$ MICLD$_{50}$ of PES were administered i.p. in rabbits and guinea pigs which had received an injection of EE-2.

*Results.* The results presented in Table I indicate that the attenuated VEE strain effectively protected mice not only against a large challenge dose of virulent VEE virus but also against a substantial dose of virulent EEE virus. The protective responses were elicited after immunization with the attenuated strain administered by the i.p., s.c., or respiratory route. As expected, the protective effect was greater when the immunized animals were challenged with the virulent homologous strain than when challenged with the heterologous EE-2 virus.

As shown in Table II, rabbits and guinea pigs, which were naturally refractory to EE-2, resisted a challenge dose of $10^{5.3}$ MICLD$_{50}$ of the virulent PES. Control animals which had not previously received an injection of EE-2 died 3 days after a PES challenge of $10^2$ MICLD$_{50}$.

Before these data were finally assessed,

TABLE II. Protection of Rabbits and Guinea Pigs against VEE Virus after Injection of Live EEE Virus.

| Challenge dose of VEE virus ($MICLD_{50}$)* | No. dead/Total No. challenged with VEE virus | | | |
|---|---|---|---|---|
| | Immunized with EEE virus | | Unimmunized controls | |
| | Rabbits† | Guinea pigs‡ | Rabbits | Guinea pigs |
| $10^{2.0}$ | | | 2/2 | 8/8 |
| $10^{5.3}$ | 0/2 | 0/10 | | |

* VEE virus challenge by intraper. route.
† $10^8$ $MICLD_{50}$ of EEE virus given intraper.
‡ $10^6$ $MICLD_{50}$ " " " " " .

however, tests were carried out to demonstrate that the virulent PES, the attenuated 9t strain which was derived from it, and EE-2 virus preparations represented separate and distinct etiologic agents. Such tests were performed to eliminate the possibility that contamination of virus strains might have been responsible for the cross-protection which was observed. In Table III, a number of distinguishing properties of each virus are listed. PES virus was lethal for mice, rabbits and guinea pigs when doses of $10^2$ $MICLD_{50}$ were given i.p. but nonlethal for 4-day chicks when doses of $10^8$ $MICLD_{50}$ were given s.c. The attenuated strain was nonlethal for mice, rabbits, guinea pigs and 4-day chicks when doses of $10^6$ $MICLD_{50}$ were given i.p. With EE-2 virus, rabbits resisted $10^8$ $MICLD_{50}$ given i.p. Guinea pigs resisted $10^6$ $MICLD_{50}$ by the i.p. route but doses of $10^7$ $MICLD_{50}$ or greater produced illness and death. A dose of $10^2$ $MICLD_{50}$ of EE-2 given s.c. was lethal for 4-day chicks. The 3 strains were also distinguishable by plaque tests with chick fibroblasts. The size of plaques produced by PES was 3-7 mm, by EE-2 it was 2-3 mm and by 9t, 0.5-1.5 mm. In addition, immune monkey sera prepared against 9t or PES neutralized both strains in tissue culture and mice but failed to neutralize EEE virus. Conversely, immune rabbit sera prepared against EE-2 virus neutralized this strain in mice but failed to neutralize 9t in tissue culture.

*Discussion.* The results of these experiments indicated that immunization of mice with the attenuated 9t strain of VEE virus protected them against a large lethal dose of the homologous virulent strain and against substantial doses of the heterologous EEE virus. Similarly, cross-protection against a lethal VEE-virus challenge was evident in rabbits and guinea pigs immunized with living EEE virus. It was necessary to test for cross-protection between VEE and EEE virus strains in at least 2 host-species because of the inherent susceptibility of mice to EE-2 and because rabbits and guinea pigs were naturally resistant to challenge with EE-2. In the mice, immunization was not readily accomplished with the live antigen while in the latter hosts it was difficult to assess an acquired resistance in animals that were inherently refractory to the challenge agent.

It is of particular importance to note that antisera from laboratory animals infected with EEE or VEE virus did not cross-react in serum-neutralization tests that were carried out in mice or in tissue cultures. In the latter system, neither an inhibition of a cpe (cell lysis) in fluid cultures nor an inhibition of formation of plaques was observed. Direct experimental evidence that explains the

TABLE III. Properties Which Distinguish Parent Egg Seed (PES) and Attenuated (9t) Strains of VEE Virus and EEE (EE-2) Virus as Distinct Etiologic Agents.

| Property | Virus strain | | |
|---|---|---|---|
| | PES | 9t | EEE |
| Lethality* for mice i.p. | + | — | + |
| " " guinea pigs i.p. | + | — | — |
| " " 4-day chicks s.c. | — | | + |
| " " rabbits i.p. | + | — | — |
| Neutralized by anti-EEE sera | — | — | + |
| " " anti-9t or anti-PES sera | + | + | |
| Plaque size on chick fibroblasts | Large | Minute | Intermediate |

* Lethality (+): doses of $10^2$ $MICLD_{50}$ were lethal by route indicated.
Lethality (—): doses of $10^6$ $MICLD_{50}$ were nonlethal by route indicated.

mechanism of the cross-protection in animals, therefore, is lacking.

*Summary.* Mice were capable of withstanding a large challenge dose of virulent Venezuelan equine encephalomyelitis virus and a substantial challenge dose of eastern equine encephalomyelitis virus after immunization with an attenuated 9t strain of VEE virus. This resistance was elicited after administration of the 9t strain by the intraperitoneal, subcutaneous, and respiratory routes, the latter being accomplished by short exposures to aerosols of the attenuated strain. Rabbits and guinea pigs, which were refractory to infections with EEE virus, were protected against lethal doses of the heterologous virulent VEE virus.

The author expresses gratitude to Dr. Peter J. Gerone and Mr. Boyd Yates for their participation in carrying out aerosol exposures.

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Received May 19, 1961. P.S.E.B.M., 1961, v107.

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# Thermal Degradation of Foot-and-Mouth Disease Virus Into Infectious Ribonucleic Acid.* (26703)

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Infectious ribonucleic acid (RNA) was first obtained from foot-and-mouth disease virus (FMDV) by phenol extraction(1,2). Mussgay(3) presented evidence that FMDV dissociates upon acidification at pH 5.0 into RNA and protein subunits. Bachrach(4) reported that FMDV, which appeared to be completely inactivated by boiling at 100°C for 5 minutes, still yielded infectious RNA upon extraction with phenol; phenol-derived RNA was not completely inactivated after heating at 85°C for 5 minutes; and (5) FMDV-RNA has a wider pH stability spectrum than FMDV preparations that contain ribonuclease (RNase).

The research reported here shows that heating degrades FMDV into infectious RNA whenever environmental RNase is simultaneously inactivated with hot sodium dodecylsulfate (SDS) or when Rnase has been previously removed.

*Materials and methods.* FMDV, type A, strain 119, which had been passaged in calf-kidney cultures 100 times, was used. Virus was concentrated 100-fold from tissue culture fluid containing 2% bovine serum by precipitation with 20% methanol at 0°C, resuspension in 1:1 Tris-Hanks' solution containing 0.5% bovine lactalbumin hydrolyzate (BLH), dialysis to remove methanol, and clarification at 15,000 rpm for 15 minutes. Such concentrates still contained RNase(6). Virus from which RNase was to be quantitatively removed was propa-

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* A preliminary report of this work was presented at the Primer Congreso Panamericano de Biol. y Patol. Exp., Caracas, Venezuela, Sept. 24-Oct. 1, 1960.

TABLE I. Effect of Heating FMDV Concentrates Containing Environmental Ribonuclease with and without Sodium Dodecylsulfate.

| | Virus treatment | | | Plating of treated virus (PFU/ml) | |
|---|---|---|---|---|---|
| Exp. No. | SDS conc. | 5-min. heating | RNase* added | Before phenol ext'ns | After phenol ext'ns |
| 1 a | .1% | none | none | $2.2 \times 10^7$ | $7.0 \times 10^2$ |
| | " | " | .5 μg | $2.2 \times 10^7$ | |
| b | .1% | 61°C | none | $3.2 \times 10^2$ | $8.5 \times 10^2$ |
| | " | " | .5 μg | 0 | |
| c | none | 61°C | none | 0 | 0 |
| | " | " | .5 μg | 0 | |
| 2 a | .1% | none | none | $3.2 \times 10^7$ | $6.0 \times 10^2$ |
| b | .1% | 61°C | none | $2.0 \times 10^3$ | $1.5 \times 10^3$ |
| | " | " | .5 μg | 0 | |
| c | .1% | 85°C | none | $7.0 \times 10^2$ | $5.5 \times 10^2$ |
| | " | " | .5 μg | 0 | |

* Infectious RNA was identified by its sensitivity to pancreatic ribonuclease (Worthington Biochemical Corp.) at 25°C for 15 min.

gated in cultures devoid of serum. Cell-derived RNase was removed by methanol precipitation in the presence of 0.03% Armour bovine serum albumin, extractions with 1:1 BuOH-$CHCl_3$ mixtures, and differential centrifugation in which virus was precipitated at 40,000 rpm for 90 minutes before resuspension in 0.01M phosphate-0.11M NaCl buffer at pH 7.5. *Infectious RNA* was produced by a modification of the phenol procedure(5), and also, as this report will show, by heating FMDV in the presence of 0.1% SDS or by heating FMDV that has been freed of RNase. *Virus was heated* in water baths. Temperatures of 61° and 85°C were reached in about 2 minutes, and heating was continued for 5 minutes longer. The tubes were then cooled quickly by immersion in an ice bath. *RNA and FMDV plaque assays* have been described previously(5,7). Dilutions for assay were made in Hanks' fluid-0.5% BLH for both virus and RNA. All platings were made on calf-kidney cultures previously washed 3 times with Hanks' fluid-0.5% BLH to remove RNase. One-tenth ml of decimally-diluted test fractions were plated, the cultures incubated at 37°C for 90 minutes, agar nutrient medium applied, and plaque-forming units (PFU) counted after 3 days' incubation at 37°C.

*Results.* Direct comparisons were first made of heating RNase-contaminated FMDV concentrates with and without 0.1% SDS (Table I). The data show that FMDV yields infectious RNA after phenol extraction (Exp. 1a, 2a); virus heated in 0.1% SDS contains infectious RNA even before phenol treatment (Exp. 1b, 2b-c); phenol treatment does not produce additional RNA in heated virus preparations (Exp. 1b, 2b-c); and virus heated without detergent does not yield infectious RNA even if subsequently treated with phenol (Exp. 1c).

It next appeared important to examine the thermal behavior of FMDV which had been processed to eliminate RNase. Its degradation by heat is summarized in Table II.

The data in Table II show that purified FMDV concentrates produced from serum-free cultures contain little or no RNase activity before (Exp. 1a) or after (Exp. 2b-c) heating and yielded infectious RNA after heating at 61° or 85°C for 5 minutes in the absence of SDS (Exp. 1b, 2b-c). The heat-derived RNA was not increased in quantity by subsequent extractions with phenol (Exp. 1b).

*Discussion.* The data show that FMDV degrades upon heating at 61° or 85°C into infectious RNA provided that environmental RNase is either inactivated by hot SDS or is removed before heating. This negates the earlier hypothesis(4) that heating FMDV at 61° to 100°C in the presence of 0.1% SDS only inactivates an infection-initiating property of its protein coat, leaving a phenol-extractable infectious core. The present studies show that it was not the intact virus

TABLE II. Effect of Heating Ribonuclease-Free FMDV Concentrates in the Absence of Sodium Dodecylsulfate.

| | Virus treatment* | | | Plating of treated virus (PFU/ml) | |
|---|---|---|---|---|---|
| Exp. No. | 5-min. heating | Acidification | RNA added (PFU/ml) | Before Phenol ext'ns† | After Phenol ext'ns |
| 1 a | none | none | none | $2.0 \times 10^7$ | $1.7 \times 10^2$ |
| | ” | pH 3.2 | ” | 0 | |
| | ” | ” | $1.2 \times 10^3$ | $1.6 \times 10^3$ | |
| b | none | none | none | $4.0 \times 10^7$ | $2.7 \times 10^2$ |
| | 61°C | ” | ” | $6.0 \times 10^2$ | $3.0 \times 10^2$ |
| | 85°C | ” | ” | $1.3 \times 10^2$ | $6.0 \times 10^1$ |
| 2 a | none | none | none | $4.5 \times 10^7$ | |
| b | 61°C | none | none | $5.5 \times 10^2$ | |
| | ” | ” | $1.8 \times 10^2$ | $8.0 \times 10^2$ | |
| c | 85°C | none | none | $5.0 \times 10^1$ | |
| | ” | ” | $1.8 \times 10^2$ | $3.0 \times 10^2$ | |

* Acidification at pH 3.2 for 3 min. and readjustment to pH 7.5 completely inactivates FMDV and RNA without inactivating RNase(5,8). RNA added was made by the phenol method.

† The entities produced from heated virus yielded no plaques after incubation with 0.1 μg pancreatic RNase at 25°C for 15 min.

which survived boiling at 100°C for 5 minutes but rather the thermally released RNA. The fact that FMDV-RNA prepared by the phenol method can withstand such boiling in 1 M NaCl without complete inactivation has been confirmed by Thomas and Leclerc (9). In addition, thermal disruption of the protein coat allowing digestion of the exposed RNA by environmental RNase tends to reveal the mechanism of the high-temperature, rapid inactivation of FMDV with its associated high energy of activation, approximately 120,600 calories per mole(10).

Heat alone degrades FMDV without the action of SDS as evidenced from the fact that FMDV without RNase present yielded infectious RNA. Moreover, FMDV is completely resistant to 2 to 5% SDS at 25° and 37°C for at least 48 and 0.5 hours, respectively(11). Also, hot SDS is a protein denaturant capable of inactivating RNase during extraction of sodium ribonucleate from tissues(12).

The thermal degradation of FMDV by heat is quite analogous to its splitting upon acidification at pH 5.0(3) in an RNase-free environment into infectious RNA and protein subunits. Consistent with Mussgay's finding is the wider pH-stability spectrum of infectious RNA, pH 4-11.5, than of FMDV containing RNase, pH 7-10(5).

*Summary.* Foot-and-mouth disease virus (FMDV) heated at 61° to 85°C yields infectious ribonucleic acid (RNA) when either sodium dodecylsulfate (SDS) is present to inactivate environmental ribonuclease (RNase) or when this enzyme is removed before heating the virus. Heat alone degrades FMDV into infectious RNA, and hot SDS only serves to inactivate RNase. Subsequent phenol treatments of heat-derived infectious RNA preparations did not significantly add to the amount of infectious RNA present.

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Received May 19, 1961. P.S.E.B.M., 1961, v107.

## Isocarboxazid Metabolism in Rat Liver Homogenate. (26704)

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Isocarboxazid (Marplan®), 1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl) hydrazine is a potent monoamine oxidase inhibitor(1) being used in treatment of various depressions(2,3, 4) and in management of angina pectoris(5, 6). In rats 75% of the radioactivity of an intraperitoneal dose of $C^{14}$-isocarboxazid labeled in the benzyl moiety was recovered in the urine in 24 hours(7). By reverse isotope dilution, virtually all radioactivity was found to reside in hippurate indicating that the benzyl moiety of isocarboxazid was oxidized to benzoate prior to formation and excretion of hippurate. The present study was designed to gain information concerning this biological conversion of the benzyl moiety to benzoate.

*Methods.* All incubations of liver homogenates were performed in a Dubnoff metabolic shaker at 38°C. Isocarboxazid was solubilized in an aqueous medium *via* Tween 80 as described by Meier *et al.*(8). For its determination, isocarboxazid was quantitatively extracted from an aliquot of the incubation medium by 2 volumes followed by 1 volume of chloroform. The combined chloroform extracts were backwashed with ½ volume of water and evaporated to dryness. The residue was then dissolved in acetone and the isocarboxazid content measured in a Beckman DU spectrophotometer by the method of Colarusso *et al.*(9). Synthesis of $C^{14}$-isocarboxazid with the methylene carbon of the benzyl moiety labeled has been described(7). Radioactivity was measured in a Nuclear of Chicago Corp. D-47 flow gas counter and corrected for self-absorption.

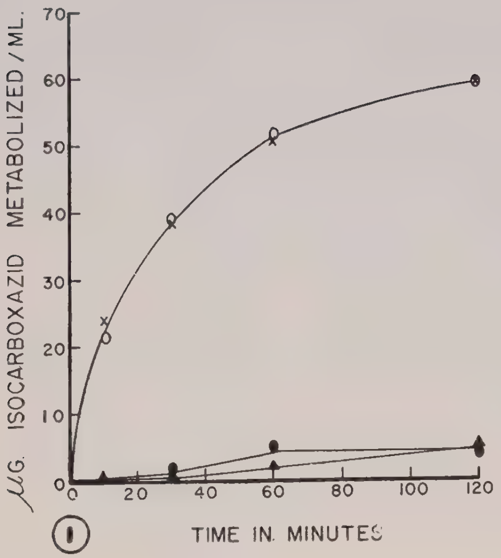
A solvent system of tertiary butanol:methanol:water (50:40:10) run by the ascending method on Whatman #1 paper pretreated with McIlvaine's phosphate-citrate buffer, pH 3.7, was employed for separation of labeled benzylhydrazine from other labeled components in extracts of the incubation medium. "Carrier" benzylhydrazine (technical grade containing 2% benzylamine) was added to the extract before it was applied to the paper and its position was determined by spraying the paper with a 1% solution of p-dimethylaminobenzaldehyde in 1N HCl. Radioactivity on the chromatograms was located by putting the papers through a Nuclear of Chicago Actigraph II chromatogram scanner.

Labeled benzylhydrazine was determined by a reverse isotope dilution technic involving the synthesis of benzylhydrazine monooxalate. For this purpose, an extract of the incubated material was prepared by adding an equal volume of absolute ethanol to an aliquot of incubation medium and filtering off the proteins after 15 minutes in an ice bath. Benzylhydrazine hydrochloride (300 mg as carrier) was dissolved in an aliquot of this extract which was then made alkaline with dilute NaOH solution, and following the addition of anhydrous sodium sulfate was extracted with 2 volumes of chloroform. The chloroform layer was concentrated to an oil after the complete removal of water by further addition of sodium sulfate. Oxalic acid (200 mg) in alcohol was added to precipitate benzylhydrazine monooxalate and this product was recrystallized from ethanol-water to constant specific activity. Furthermore, the final product caused no depression in the melting point of authentic benzylhydrazine monooxalate.

*Results.* 1. Enzymatic destruction of isocarboxazid. The aerobic disappearance of isocarboxazid in presence of rat liver homogenate and boiled rat liver homogenate is presented in Fig. 1. After 2 hours about 90% of the isocarboxazid was destroyed by the liver homogenate while the breakdown in presence of boiled liver homogenate occurred to the same small extent as found in the buffer control. This apparently enzymatic destruction of isocarboxazid was checked by using ethyl alcohol instead of heat to denature the enzymes. Furthermore, the effect of a casein suspension on the disappearance of isocarboxazid was investigated to ascertain whether this disappearance was catalyzed by specific enzymes or by non-specific proteins. Also included in this experiment was a study of the effect of anaerobiosis on liver homogenate-catalyzed isocarboxazid destruction (Table I). While there was some destruction of isocarboxazid in the buffer alone (9 and 29%), 92% disappeared in presence of liver homogenate. Denaturation of enzymes with ethanol resulted in a decrease in isocarboxazid destruction to the buffer control levels, and casein could not replace the homogenate as a catalyst. Furthermore, the anaerobic incubation of isocarboxazid with liver homogenate resulted in a breakdown almost identical to that seen aerobically. It is concluded from these experiments that in the system employed isocarboxazid was metabolized by liver enzymes and that the first step in this metabolism was not an oxidation.

The effect of substrate concentration on the metabolism of isocarboxazid is shown in Fig. 2. It is apparent that the reaction is linear with respect to substrate concentration up to approximately 0.5 x $10^{-3}$ M (116 $\mu$g/ml).

2. Isolation of intermediates in isocarboxazid metabolism using $C^{14}$-isocarboxazid (labeled benzyl moiety). Since enzymatic hydrolysis of isocarboxazid might lead to the formation of benzylhydrazine, its presence in incubation medium extracts was investigated by paper chromatography and reverse isotope dilution. Radioactive material in the same position as the "carrier" benzylhydrazine was found in chloroform and ethanol



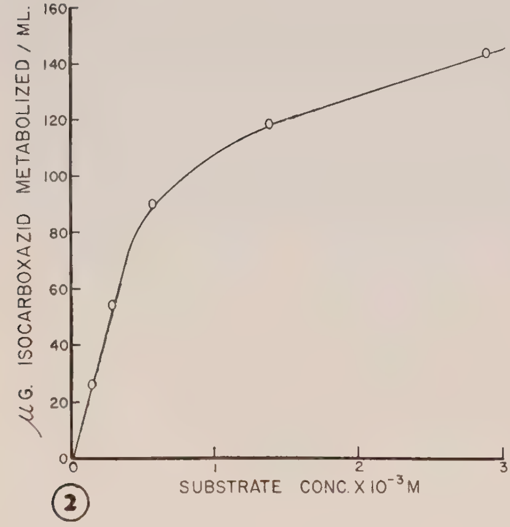


FIG. 1. Metabolism of isocarboxazid as a function of time. Each flask contained 400 $\mu$g of isocarboxazid dispersed in Krebs-Ringer phosphate buffer, pH 7.4 (1 ml). Symbols refer to addition of rat liver homogenate as follows: (○) and (×), duplicate flasks containing 2 ml of 1:4 liver homogenate in Krebs-Ringer phosphate buffer; (●), 2 ml of boiled homogenate (3 min. in boiling water bath); and (▲), no homogenate added. Volume in each flask was brought to 6 ml with buffer, making final isocarboxazid concentration 67 $\mu$g/ml.

FIG. 2. Effect of substrate concentration on isocarboxazid metabolism. Duplicate flasks containing 2 ml of 1:4 liver homogenate in Krebs-Ringer phosphate buffer, pH 7.2, and 4 ml of buffer containing graded amounts of isocarboxazid were incubated aerobically for 2 hr at 38°C. Values for isocarboxazid metabolized were corrected for the slight loss of isocarboxazid observed in incubated control flasks containing just isocarboxazid and buffer.

TABLE I. Effect of Liver Homogenate and Casein Suspension on Isocarboxazid Destruction; Effect of Alcohol and Anaerobiosis on Homogenate-Induced Destruction.

| Sample | Contents | % isocarboxazid destruction after 2 hr at 38°C |
|---|---|---|
| 1—Aerobic | Isocarboxazid + buffer | 29 |
| 2 " | *Idem* | 9 |
| 3 " | Isocarboxazid + homog. | 92 |
| 4 " | *Idem* | 92 |
| 5 " | Isocarboxazid + homog. + EtOH | 8 |
| 6 " | *Idem* | 12 |
| 7 " | Isocarboxazid + casein | 28 |
| 8 " | *Idem* | 15 |
| 1—Anaerobic | Isocarboxazid + homog. | 91 |
| 2 " | *Idem* | 91 |

Isocarboxazid concentration in each flask was $3.6 \times 10^{-4}$ M; final volume was 3 ml. Isocarboxazid, rat liver homogenate and casein were prepared in Krebs-Ringer phosphate buffer, pH 7.3. One ml of homogenate (1:4), 1 ml of absolute ethanol, and 1 ml of a 5% suspension of casein were employed where noted in the table. These additions replaced an equal volume of buffer. Evacuated Thunberg tubes were used for anaerobic incubation.

extracts of the incubation medium when these extracts were chromatographed as described under *Methods*. The use of "carrier" benzylhydrazine made identification of labeled benzylhydrazine possible in spite of the fact that there was a wide variation in $R_f$ values of benzylhydrazine ($R_f$ .30-.47). In any one sample the difference in $R_f$ values between labeled component and added benzylhydrazine did not exceed 0.02. The variation of the benzylhydrazine $R_f$ values might be due to the effects of other components in these extracts or to sensitivity of this chromatographic system to factors which were not adequately controlled.

The presence of labeled benzylhydrazine in the incubation medium was confirmed by reverse isotope dilution (Table II). Approximately 80% of the radioactivity was extracted with alcohol following both aerobic and anaerobic incubation (column a) while 91% was extracted from the control flask containing boiled homogenate (flask 5). From the specific activities of the isolated benzylhydrazine monooxalate, amount of benzylhydrazine hydrochloride used as carrier, and radioactivity of the alcohol extract aliquot to which carrier was added, the percentage of the total extracted radioactivity residing in benzylhydrazine was calculated (column b). These values ranged from 48.3% to 52.0% and were independent of the mode of incubation. Furthermore, in the control flask only an insignificant fraction of the extracted radioactivity (0.4%) was calculated to be benzylhydrazine. The values of column (b) can be taken as the percentage conversion of isocarboxazid to benzylhydrazine by assuming the benzylhydrazine content of that radioactivity not extracted by alcohol (100%-column a) to be the same as the percentage benzylhydrazine in the radioactivity which was extracted (column b). This asumption appears valid since in the control flask (flask 5) 9% of the radioactivity was not extracted with alcohol while when benzylhydrazine was formed (flasks 1-4) approximately 20% of the radioactivity was not extracted, indicating that roughly half of this unrecovered radioactivity was benzyl-

TABLE II. Percentage of C$^{14}$-isocarboxazid Converted to Labeled Benzylhydrazine after 2 Hour Incubation with Rat Liver Homogenate.

| Flask No. | Incubation | % incubation medium radioactivity in alcohol extract (a) | Specific activity of isolated benzylhydrazine monooxalate (cpm/mg) | % alcohol extract radioactivity present in benzylhydrazine[*] (b) |
|---|---|---|---|---|
| 1 | Aerobic | 77 | 416 | 48.3 |
| 2 | Aerobic | 78 | 444 | 52.0 |
| 3 | Anaerobic | 82 | 453 | 50.0 |
| 4 | Anaerobic | 83 | 467 | 49.5 |
| 5[†] | Aerobic | 91 | 2 | 0.4 |

[*] These values also represent the % conversion of isocarboxazid to benzylhydrazine (see text).

[†] Flask 5 contained boiled rat liver homogenate and served as a control.

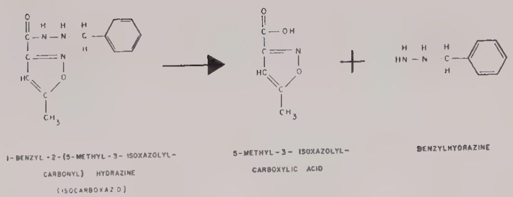

1-BENZYL-2-(5-METHYL-3-ISOXAZOLYL-CARBONYL) HYDRAZINE (ISOCARBOXAZID)
5-METHYL-3-ISOXAZOLYL-CARBOXYLIC ACID
BENZYLHYDRAZINE

FIG. 3. Primary reaction in *in vitro* metabolism of isocarboxazid.

hydrazine. This agrees with the roughly 50% of the alcohol extract radioactivity recovered as benzylhydrazine (column b).

*Discussion.* It has been demonstrated *in vitro* that rat liver homogenate enzymatically catalyzes the breakdown of isocarboxazid and that one major product of this metabolism is benzylhydrazine. These findings can most simply be interpreted as reflecting a hydrolytic cleavage of isocarboxazid to yield 5-methyl-3-isoxazolylcarboxylic acid and benzylhydrazine (Fig. 3). The isolation of benzylhydrazine from animal tissues following administration of pharmacological doses of isocarboxazid has not been reported. However, Roth and Rieder† have detected benzylhydrazine in rat and guinea pig tissues following massive doses of isocarboxazid. This, in conjunction with the present finding that *in vitro* there is extensive metabolism of isocarboxazid to benzylhydrazine and the previous finding(7) that *in vivo* 75% of the drug was cleaved with the benzyl moiety further oxidized to benzoate and excreted as hippurate in 24 hours, indicates that benzylhydrazine is an intermediate in the metabolism of isocarboxazid *in vivo*. Since benzylhydrazine is a more potent MAO inhibitor than isocarboxazid, the role of benzylhydrazine as an agent responsible for various aspects of the pharmacological activity of isocarboxazid becomes of prime importance. This problem is currently being studied.

*Summary.* Isocarboxazid was extensively metabolized under aerobic or anaerobic conditions in an *in vitro* system containing rat liver homogenate as the enzyme source. Benzylhydrazine was a major product of this metabolism as determined by paper chromatography and reverse isotope dilution in studies with $C^{14}$-isocarboxazid. The possible occurrence of this pathway *in vivo* was discussed.

The author wishes to acknowledge the very competent technical assistance provided by Mr. E. Postma.

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Received February 6, 1961. P.S.E.B.M., 1961, v107.

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† Personal communication from Hoffmann-La Roche, Inc., Basel, Switzerland.

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# Development of Serum Haptoglobins in Man.* (26705)

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(Introduced by Paul Reznikoff)
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Twenty years ago Polonovski and Jayle described a fraction of serum protein capable of combining with hemoglobin to form a stable complex which possessed peroxidase activity. This fraction they called haptoglobin (1). Smithies, using the technic of starch gel electrophoresis, has demonstrated that several haptoglobins may be present in a single serum, and that there are at least 3 different genetically determined haptoglobin patterns(2). Tuttle reported that haptoglobins were not present in the blood of newborns and did not appear until after the first few weeks of life(3). Smithies observed that haptoglobins were not always present in sera of young children(2). Bearn and Franklin reported that antisera prepared from electrophoretically isolated haptoglobin complexes failed to react with umbilical cord sera, but did react with normal sera and isolated haptoglobin complexes(4). This may be interpreted as indicating the absence of haptoglobins in cord sera. On the other hand, Galatius-Jensen was able to determine haptoglobin patterns in 4 out of 34 samples of umbilical blood from newborns, and in sera of 6 of 10 children 8 days of age whose haptoglobins were not detectable at birth (5). Nymen, too, has recently reported that she demonstrated haptoglobins in 11 of 79 samples of cord blood from newborns, and could find haptoglobins in the sera of 3 infants, 2, 12, and 14 days old(6). Our preliminary investigations indicated that apparently the capacity for producing haptoglobins does not develop until some time after birth(7). In the present study we have attempted to determine more accurately the time of appearance of haptoglobins in humans.

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* Supported by research grants from Nat. Cancer Inst., U.S.P.H.S.

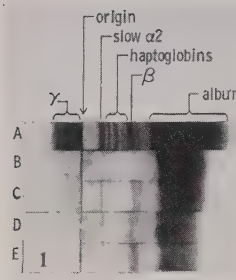
*Materials and methods.* Blood was collected from the pulsating hearts of 5 fetuses, age 14, 14, 18, 18, and 23 weeks, from the umbilical cords of 61 newborn including 10 delivered by Caesarian section, and from a peripheral vein or the heel of 86 infants through 15 weeks of age. Haptoglobins were analyzed by zone electrophoresis of the serum in starch gel(8). Gels were prepared from a single batch of Connaught prehydrolyzed starch (Lot #124) using a concentration of 12.5 g of starch per 100 cc of 0.021 M borate buffer of pH 9.05. Electrophoresis was carried out at 10°C for 16 hours with a potential of 4.5 volts/cm. After electrophoresis, the interiors of the gel strips were exposed by slicing them longitudinally. One-half was stained with naphthalene black B 200 to determine the distribution of serum proteins present in the sample. The other half was stained with ortho-tolidine according to the method of Kohn and O'Kelly(9) to specifically detect haptoglobin-hemoglobin complexes and/or free hemoglobin. Except for some of the cord sera, each serum sample was run in duplicate, with and without addition of normal human hemoglobin at a final concentration of 150 mg %. Thus each sample was tested twice; once free of hemoglobin and once with hemoglobin added. When haptoglobins were present, the sample with the hemoglobin added showed a change in mobility of the bands in the haptoglobin region after staining with naphthalene black. After staining with ortho-tolidine, only the hemoglobin-haptoglobin complex and in some instances the excess free hemoglobin appeared. When haptoglobins were absent, only a free hemoglobin band was found(10).

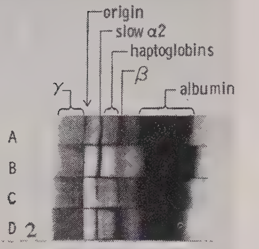
*Results.* Haptoglobins were not detectable in the 5 fetal sera, and when hemoglobin was added to the samples, only a free hemoglobin band appeared. A single band present in the haptoglobin region of one sample when it was run initially, disappeared when it was run with the hemoglobin added (Fig. 1). We have found haptoglobins in 10% of the 63 cord bloods tested. Forty of them were compared to the corresponding maternal pattern, and the most significant differences were found in the haptoglobin region. In all but 6 cord sera, haptoglobins were absent, whereas all maternal patterns showed haptoglobins (Fig. 2). Haptoglobins when present in the cord sera were in low concentration as determined by visual evaluation. None of the 10 cord bloods obtained from newborns delivered by Caesarian section contained haptoglobins. The results of the electrophoretic study of the serum proteins of the 86 infants (Table I) strongly indicated that while in most instances appreciable amounts of haptoglobins may not be present at birth, they do appear as early as 12 hours after birth, are present in 75% of the infants during the first 2 weeks, and were found in 100% of the infants tested by the beginning of the third week. Where it was possible to make a serial study of the same child, the progressive increase in amount of haptoglobins with age could readily be seen (Fig. 3).

*Discussion.* From these observations we conclude that haptoglobins are rarely present at birth, and then only in low concentrations. This absence or low concentration of haptoglobins may be attributed either to the low rate of synthesis in the newborn, or to the fact that large numbers of red blood cells are destroyed shortly after birth, resulting in liberation of excessive amounts of hemoglobin into the plasma causing removal of the haptoglobins faster than they can be synthesized. The role played by each of these mechanisms requires further study. It seems likely that in some infants haptoglobin synthesis begins before birth, and that by the age of 2 weeks virtually all infants are able to produce haptoglobins.

Other proteins are known to develop at or shortly after birth in animals. For example, an abrupt increase in activity of tyrosine-alpha-ketoglutarate transaminase in rat livers from the second to the twelfth hour after birth has been demonstrated(11). Haptoglobin synthesis in the human precedes by a wide margin the development of antibody producing activity(12). The appearance of haptoglobins, though shown to occur somewhat earlier than previously reported, does not seem to parallel the development in man of adult hemoglobin, with which it is closely associated(13).

Definitive information is lacking regarding the site of synthesis of the haptoglobins. Lack of correspondence in the appearance of haptoglobins with development of active immune response suggests that the antibody producing cells are not a site of haptoglobin synthesis. On the other hand, the liver has not been eliminated from consideration by the older studies which utilized less sensitive electrophoretic methods. Although electrophoretic studies have indicated that at time of birth the liver is capable of synthesizing all of the major plasma fractions except gamma globulin(14), minor components can





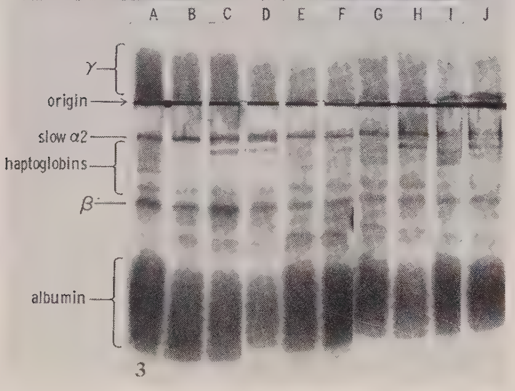


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FIG. 1. Starch gel electrophoretic patterns of fetal serum proteins. *Key:* A, Adult serum. B, Fetal serum (18 wk, 16.5 cm). Proteins are seen in albumin, beta globulin, slow alpha$_2$ globulin and gamma globulin regions. Note band in haptoglobin region. C, Fetal serum (B with 150 mg % hemoglobin added). Same pattern as in B except that the band in haptoglobin region is absent and a free hemoglobin band appears just faster than beta. D and E, Fetal sera (14 wk, 11.5 cm). Pattern same as in older fetus B except for absence of gamma globulin. No evidence of haptoglobins in either sera.

FIG. 2. Starch gel electrophoretic pattern of maternal and corresponding cord serum proteins. *Key:* A, Maternal serum. B, Umbilical cord serum from child of A. No evidence of haptoglobins. All other serum proteins present. C, Maternal serum. D, Umbilical cord serum from child of C, showing haptoglobins present.

FIG. 3. Series of starch gel electrophoretic patterns of serum proteins of same child from 2 days through 22 days of age, showing progressive increase in amount of haptoglobin. *Key:* A, Adult serum (haptoglobin type 2-1). B, Infant serum (2 days) with 150 mg % hemoglobin added. There is a faint trace of haptoglobin present. C, Infant serum with 3+ hemolyses (5 days). Haptoglobins can now readily be seen. D, Infant serum, 4+ hemolyses (7 days). No change from previous haptoglobin pattern. E, Infant serum (13 days). F, Infant serum (13 days) with 150 mg % hemoglobin added. The haptoglobin pattern has become more complex with additional faster bands appearing. G, Infant serum (14 days). Still more intense pattern as compared to E. H, Infant serum with 150 mg % hemoglobin added (14 days). Haptoglobin bands now very intense. I and J, Infant serum (22 days) with no hemoglobin and 150 mg % hemoglobin added respectively. A still more intense haptoglobin pattern is shown which remains at this level as the child increases in age. The haptoglobin type is now readily discernible and can be classified as the homozygous 2-2 type.

TABLE I. Chronology of Appearance of Haptoglobins in Fetal, Newborn, and Infant Sera as Indicated by Starch Gel Electrophoresis after Staining with Ortho-Tolidine and Naphthalene-Black B-200.

| | | No. with haptoglobins present | | | |
|---|---|---|---|---|---|
| | | Detected with | | | |
| Age | No. of samples | Both ortho-tolidine and naphthalene black | Ortho-tolidine stain only | Naphthalene-black stain only | % of samples containing haptoglobins |
| Fetal | 3 | 0 | | | 0 |
| Cord | 63 | 6 | | | 10 |
| Day of birth | 2 | 1 | | | 75 |
| 1 day | 3 | | 2 | | |
| 2 days | 5 | 4 | | 1 | |
| 3 ” | 3 | 3 | | | |
| 4 ” | 6 | 5 | | | |
| 5 ” | 6 | 5 | | | |
| 6 ” | 3 | 2 | | | |
| 7 ” | 1 | 1 | | | |
| 2nd wk | 7 | 4 | | | |
| 3rd wk | 3 | 3 | | | 100 |
| 4th ” | 6 | 6 | | | |
| 5th ” | 3 | 2 | | 1 | |
| 6th ” | 3 | 2 | 1 | | |
| 7th ” | 10 | 9 | 1 | | |
| 8th ” | 6 | 6 | | | |
| 9th ” | 4 | 4 | | | |
| 10th ” | 4 | 4 | | | |
| 11th ” | 2 | 2 | | | |
| 12-15th wk | 9 | 9 | | | |

be detected only by more sensitive methods.

*Summary.* To determine the time of appearance of the haptoglobin serum proteins in the developing human being, starch gel zone electrophoresis was performed on sera from 5 fetuses, 63 umbilical cord bloods, and 86 infants. Haptoglobins were absent in sera from the fetuses, but present in 10% of the umbilical cord bloods at birth, in 75% of the sera from infants up to 2 weeks old, and in 100% of the sera from the infants over 2 weeks of age.

For cooperation and assistance in securing the cord and newborn bloods necessary for this study, we wish to thank Dr. Marion Erlandson and Dr. Margaret Hilgartner, Dept. of Pediatric Hematology, New York Hospital, Dr. Roy Bonsnes and Miss Dorothy Wendel, Central Laboratories of New York Hospital, and Dr. Wha Shim Han, Bellevue Hospital. We also wish to thank Dr. Bartholomeus Hoogstraten, Mt. Sinai Hospital, for obtaining the fetal bloods. Dr. German Castillo, Univ. of the Philippines, participated in the early phases of the study.

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Received March 9, 1961. P.S.E.B.M., 1961, v107.

# Influence of Antibiotics and Hemorrhage upon Serum Properdin Titers.*

(26706)

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During the past decade, Fine and his collaborators have adduced increasing experimental evidence which suggests that bacterial endotoxins play a significant role in hemorrhagic shock(1). They have demonstrated that an increase in survival rate resulted from pre-treatment with antibiotics (2). On the other hand, when the reticuloendothelial system (RES) is blocked, antibiotics given by gavage may have a lethal effect(3). They have postulated that, under these conditions, antibiotics liberate increased quantities of endotoxin from the Gram-negative intestinal flora.

The present study was undertaken to test this hypothesis. It has been demonstrated that, after a certain interval of time, injections of endotoxin increase serum properdin titers(4). In a series of experiments, the effect of orally administered antibiotics upon serum properdin titers was ascertained, to observe whether serum properdin titers would also rise under these conditions. In addition, these animals were subjected to hemorrhagic hypotension to determine the effects of this antibiotic regimen upon survival rate and upon changes in properdin titre associated with hemorrhage.

*Method.* Thirty mongrel dogs weighing from 9-16 kg were selected and randomly divided into 3 equal groups. Group 1 received no treatment prior to hemorrhagic hypotension. Group II was given 2 oz. milk of magnesia orally for 5 days, starting 7 days before hemorrhage. Group III also received milk of magnesia for 5 days, but in addition was treated with 6 g of sulfasuxidine and 4 g of neomycin sulfate orally for the 7 days prior to hemorrhage.

Estimations of the viable Gram-positive organisms in the fecal flora were made on enriched nutrient agar containing 0.2 g of sodium azide per liter. For the Gram-negative organisms, Levine's E.M.B. agar (Difco) was used. A loop 1 cm in diameter was used to streak serial 10-fold dilutions of the feces on the surface of the agar plates. Observations were made of the highest dilution showing growth and whether or not the metallic sheen typical of *E. coli* was present on the E.M.B. plates. Streaks were made prior to initiation of treatment and before induction of hypotension.

The dogs were subjected to hemorrhagic hypotension in random sequence from Groups I, II and III. All animals were deprived of food but not water for 15 hours prior to experiment. Sodium pentobarbital (30 mg/kg) was given intravenously. All apparatus was sterile and incisions were made using aseptic technic. A femoral artery and vein were cannulated, and heparin‡ (2 mg/kg) was administered. The artery was connected by means of a T-tube to a mercury manometer and a collecting reservoir. The zero level of the manometer was set at the hydrostatic level of the femoral artery. The dog was allowed to bleed until its arterial pressure fell to 35 mm Hg, which was established by the height of the reservoir. After 3 hours of hypotension, the blood remaining in the reservoir was returned to the dog, and the incision was repaired.

Blood for properdin determinations was drawn before treatment and periodically throughout the experiment. Plasma was stored at –30°C in sealed vials. Properdin titers were determined by the phage neutralization method(5) and are expressed in terms of per cent of control value, as mea-

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* This study was supported by grants from U.S.P.H.S. and from Cleveland Area Heart Soc.

† Work carried out in partial fulfillment of the requirements for the M.D. degree, School of Medicine, Western Reserve Univ.

‡ Supplied thru the generosity of Dr. W. R. Kirtley of Eli Lilly Laboratories.

sured in 50% neutralization units/ml of undiluted plasma.[§]

*Results.* The 2 control groups—I, untreated, and II treated with milk of magnesia —had 4 and 5 survivors, respectively, for a total of 9 survivors out of 20. In Group III, treated with milk of magnesia plus antibiotics, 8 out of 10 survived, giving a statistically significant increase in survival rate ($P = 0.05$) as compared to the 2 control groups.

Examination of the fecal streaks made before treatment revealed Gram-positive and Gram-negative bacteria. Typical *E. coli* were present in the feces in all animals. Milk of magnesia alone reduced the growth by approximately 10 to 100-fold, but failed to eliminate *E. coli* in any of the animals in Group II. Group III, which received antibiotics in addition, showed a considerable diminution in fecal flora. An average of $10^3$ to $10^4$-fold reduction in both Gram-positive and Gram-negative growth was noted. *E. coli* were absent in all but one animal, and in this dog, the Gram-negative organisms had been reduced from growth in the $10^{-7}$ dilution before treatment to the $10^{-2}$ dilution after antibiotics.

The course of properdin titers in the animals in Group I was similar to that previously reported for untreated dogs(6,7). During the oligemic period, properdin levels remained relatively unchanged. In the first 5 hours after hypotension, titers fell rapidly, and to a greater extent in the animals that died. In the survivors, titers began to rise by the 2nd day and reached a level well above control in the following 2-4 days. Later they started to fall toward control. The average control properdin titers of the 4 survivors and 6 non-survivors were vitually identical. In fact, if all the dogs included in this study are divided into survivors and non-survivors, there is no statistical relationship between control properdin levels and survival; *i.e.*, 17 survivors, 28.0 ± 14.0 (S.D.), and 13 non-survivors, 33.1 ± 9.0 $Ph.N_{50}$ units/ml of plasma.

In Group II, treated with milk of magnesia, at the time of induction of hypotension, the 5 survivors had properdin titers averaging 140 ± 12% of their pre-treatment levels. The 5 non-survivors had an average titer just prior to oligemia of 96.2 ± 8.3% of control ($P<0.001$).

Prior to oligemia, the 8 survivors of Group III (milk of magnesia plus antibiotics) showed an average increase in properdin level to 148.5% of control titer whereas the 2 non-survivors averaged 96%. The individual properdin titers at that time were 128, 80, 255, 124, 180, 133, 122, and 150% for survivors, and 112 and 80 per cent for non-survivors. The course of properdin levels in survivors of Groups II and III after hemorrhage followed a pattern similar to that described above.

*Discussion.* The decline of properdin levels during hemorrhagic shock was first noted by Frank *et al.*(8). Subsequently it was demonstrated(6,7) that properdin titers remain unchanged during the oligemic period but decline rapidly in the hours following transfusion, and to a greater extent in non-survivors than in survivors. After the first 24 hours, properdin titers rise to levels well in excess of control, reach a peak at the 4th to 7th day, then gradually return toward control. Injections of zymosan were shown to increase significantly properdin levels as well as survival rates(9).

In the present study, as in that reported by Schweinburg *et al.*(2), treatment with antibiotics (Group III) was successful in significantly increasing the survival rate of dogs subjected to a standard hemorrhagic hypotension. The recent work of Wiznitzer *et al.*(10), relating the size of the intraintestinal pool of endotoxin to development of irreversible hemorrhagic shock, could account in part for the survivals. Seven of the 8 survivors in this group had pronounced increase in properdin titers following antibiotic administration, again demonstrating a direct relationship between increased properdin levels and survival(9), though not necessarily a cause and effect relationship.

The augmentation of properdin levels which occurred as a result of antibiotic therapy constitutes supporting evidence for the hypothesis advanced by Fine and his collaborators(3). These investigators postulated that the lethal effects produced by antibiotics administered by gavage to animals in which the RES had been blocked are attributable to relase of endotoxin from the normal intestinal flora. It has been shown that injections of endotoxin induce increased properdin titers after an initial decline(4). It is not unlikely, therefore, that in the present study the antibiotics caused increased quantities of endotoxin to be released into the intestinal tract. This in turn would, after passage through the mucosa, produce the observed elevation of the properdin titer. Antibiotics probably exert effects other than their bactericidal activities, however. For example, it has been claimed that antibiotics abrogate the depressant action of endotoxin upon the RES(11). Thus, it is conceivable that the observed changes in properdin might be ascribable to some side-action of the antibiotics.

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[§] The authors wish to express thanks to J. L. Barlow for supplying $T2r^+$ phage.

In Group II, the increase in properdin titers resulting from treatment with milk of magnesia alone is also difficult to explain. The milk of magnesia may also have released increased quantities of endotoxin, or it may have affected mucosal permeability to endotoxin, or it may have acted by some entirely different mechanism.

*Conclusion.* In dogs subjected to a standard hemorrhagic hypotension, survival rate was significantly increased by prior treatment with antibiotics. This regimen eliminated typical *E. coli,* and markedly reduced the Gram-negative fecal flora. Seven of 8 of these survivors had significantly increased properdin titers following antibiotic administration. In a group of dogs treated with milk of magnesia, though the survival rate was not increased, the survivors all had significantly increased properdin titers. The pre-treatment properdin titers were not significantly related to subsequent survival from standard hemorrhagic hypotension.

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Received March 20, 1961. P.S.E.B.M., 1961, v107.

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# Transfer of Water Across the Perfused Umbilical Cord.* (26707)

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An appreciable proportion of the water exchange of the amniotic fluid takes place through the intermedium of the fetus(1). The mechanism and site of this exchange is unknown. Since micturition and deglutition cannot account for the magnitude of this exchange, a transfer across the umbilical cord and possibly the fetal surface of the placenta has been suggested as an alternative explana-

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* This investigation was supported by a grant-in-aid from Assn. for the Aid of Crippled Children, New York.

TABLE I. Composition of Solution Representing the Amniotic Fluid Compartment.

| | g/100 ml |
|---|---|
| Sodium chloride | .620 |
| Potassium " | .037 |
| Sodium bicarbonate | .168 |
| " phosphate (buffer) | .100 |
| Urea | .027 |
| Glucose | .060 |
| Human serum albumin | 1.200 |

tion(2). It is conceivable that water, electrolytes and metabolic products may pass through Wharton's jelly at a sufficiently rapid rate to account for part of the turnover of the constituents of the amniotic fluid.

To test this hypothesis preliminary experiments were designed and carried out wherein the transfer rate of water was measured by means of radioactive tracers. Sections of human cord were perfused with Group O, Rh negative blood under a pulsating flow while the organ was immersed in a solution, the composition of which approximated that of amniotic fluid. The preparation represents a closed 2-compartment system in steady state, suited for a mathematical interpretation of tracer curves.

*Experimental.* Fresh human cords were obtained from the delivery floor. The cords were clamped while still pulsating in order to insure distention of the arteries which facilitated the subsequent cannulation. They were placed in warm Ringer's solution and the vessels on one end cannulated within less than 10 minutes after delivery. The clamps were removed and blood drained to remove all clots. The arteries were distended with a few ml of heparinized Group O blood and cannulas inserted in the opposite ends. The 2 arteries were then connected to the vein in such fashion that the direction of flow in arteries and veins was in an opposite direction. The open arterial ends were connected to a "roller pump" and the vein connected with an oxygenating system described by Nyberg, Westin and Enhorning(3). The oxygenated blood was then fed into the pump, completing the cycle. Total volume of circulating blood was 480 ml.

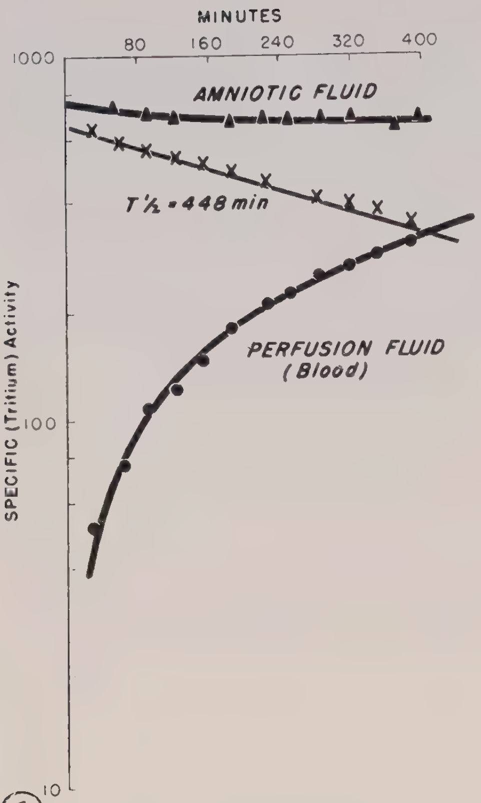
The system was briefly tested for leaks and the cord immersed in a synthetic amniotic fluid, the composition of which is given in Table I. The container was completely filled with fluid (5100 ml), sealed with silicone grease and tightly closed with numerous set screws. Provisions were also made for continuous pressure measurements, thermostatic control, removal of samples and agitation without disturbing the continuity of the system.

The perfusion was then started by setting the pump and oxygenator in motion and balancing their output. Because of the limitation of the oxygenation system, perfusion rate was set at 180 ml/min with a pulse rate of 162 beats per minute. The system was tested for at least one hour during which time equilibrium was established. Three to 4 millicuries of tritiated water were then injected into the blood or amniotic fluid compartment and samples withdrawn at frequent intervals. The samples were collected in melting point tubes and sealed. The tritium analyses were performed by subliming one drop of blood or amniotic fluid into the conversion apparatus of Graff and Rittenberg (4) and radioactivity determined according to Glasscock(5) using Bernstein-Ballantine counters in the proportional region. The results, expressed as specific activity, were then plotted on a semi-log scale and the curves analyzed by standard methods. Two examples are reproduced in Fig. 1 and 2 where the conditions were identical (length of cord, flow rate, volume). In the former (Fig. 1) the tracer was injected into the blood compartment, in the latter (Fig. 2) the amniotic fluid served as the primary compartment.

*Calculation of transfer rates.* The specific activity curves reproduced can be expressed by exponential equations of the form:

$$\begin{array}{l}\text{Amount of tracer in the ith}\\ \quad\text{compartment at time t}\end{array} = A_{ij}\, e^{-\alpha_j t} + A_{eq.}$$

Subtracting the amount of tracer at equilibrium ($A_{eq.}$) from each experimentally determined point, a straight line is obtained on a semi-log plot, the slope of which determines the value of $\alpha$. For the curves reproduced in Fig. 1, the value of $\alpha$ is 0.00154 min$^{-1}$, or a $t_{1/2}$ of 448 minutes. If total amount of tracer injected into the primary compart-



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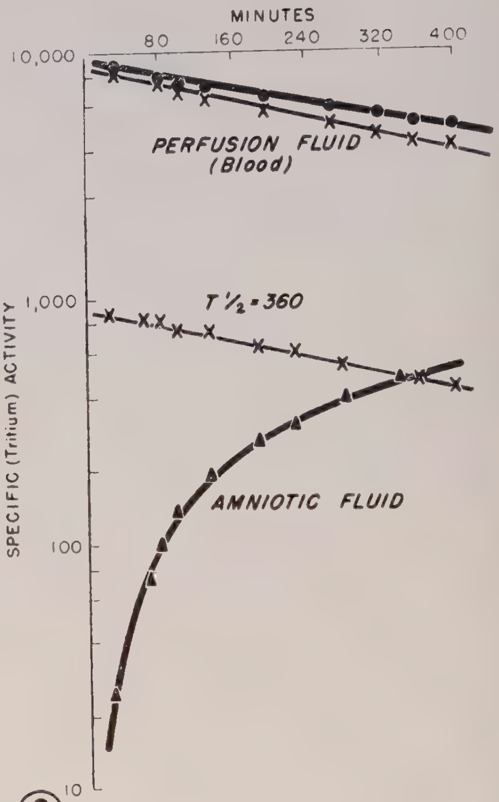

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FIG. 1. Specific tritium activity (counts/min./mM × $10^2$) in amniotic (▲) and perfusion fluid (●) as a function of time. Tracer was inj. into amniotic fluid compartment. Subtracting equilibrium value from each experimental point gives the straight line (×) which determines $t_{½}$.

FIG. 2. Specific tritium activity (counts/min./mM × $10^2$) in amniotic (▲) and perfusion fluid (●) as a function of time in min. Conditions were identical to those described in Fig. 1, but tracer was inj. in perfusion fluid.

ment is 1, the fraction remaining in this compartment declines and reaches an equilibrium value of 0.914. The fraction of tracer in the amniotic fluid (AF) at any time t is then:

$$(\mathrm{HTO})_{\mathrm{AF}} = 0.086\ e^{-0.00154\,t} + 0.914 \ldots\ldots(2)$$

Simultaneously, the amount of tracer in the secondary compartment (perfusion fluid) rises and the fraction of tracer present at equilibrium will be 0.086 of total amount injected. The experimental curve for the perfusion fluid (PF) in Fig. 1 can be described by the equation:

$$(\mathrm{HTO})_{\mathrm{PF}} = 0.086\ e^{-0.00154\,t} + 0.086 \ldots\ldots(3)$$

Equations (2) and (3) uniquely define this system. The transfer rates can be calculated according to Berman and Schoenfeld (6), using the matrix notation:

$$[l] = [A]\,[a]\,[A]^{-1}$$

or, $$\begin{bmatrix} l_{11} & l_{12} \\ l_{21} & l_{22} \end{bmatrix} =$$

$$\begin{bmatrix} 0.086 & 0.914 \\ -0.086 & 0.086 \end{bmatrix} \begin{bmatrix} 0.00154 & 0 \\ 0 & 0 \end{bmatrix} \begin{bmatrix} 1 & -10.62 \\ 1 & 1 \end{bmatrix}$$

$$= \begin{bmatrix} 1.32 & -14.06 \\ -1.32 & 14.06 \end{bmatrix} \times 10^{-4}$$

$l_{21}$ is the fraction transferred from compartment 1 (amniotic fluid) to compartment 2 (perfusion fluid) and $l_{12}$ the transfer in the reverse direction. To obtain the amount transferred per unit of time the $l$'s must be multiplied by their respective compartmental size

$$(1.32)(10^{-4})(5100) = (14.06)(10^{-4})(480) = 0.675 \text{ ml/min.}$$

or an exchange rate of 40.5 ml/hr. A calculation performed in an analoguos manner using the data of Fig. 2 where the tracer was injected into the perfusion fluid, gives an exchange rate of 50.5 ml/hr.

*Discussion.* That various organic and inorganic substances can traverse the cord has been shown by Fehling(7) and Runge, Baur and Hartmann(8). When the vein of an isolated segment of human cord was perfused with acacia-thyrode solution containing various organic and inorganic dyes, these dyes appeared in the surrounding fluid within 20 to 30 minutes. Perfusion of the arteries failed to produce the same effect. Both authors considered but could not prove that large quantities of water are exchanged in a similar manner. Fehling (l.c.) also made an unsuccessful attempt to correlate the length of the cord with the amount of amniotic fluid.

The appearance of the radiotracer in the secondary compartment and its disappearance from the primary compartment constitutes adequate evidence that an exchange of water takes place. In the present perfusion system the only possible site of this exchange is the surface of the cord, *i.e.,* across vessel walls, Wharton's jelly and umbilical amnion. The amount of water exchanged is appreciable and under the conditions prevailing *in utero* this may well account for one-half of the water turnover of the amniotic fluid.

The length of the cord, *i.e.,* the surface available for the exchange, pressure differentials and rate of perfusion but not the volume of the amniotic fluid will be a function of the transfer rate. In the present experiments, these factors were kept constant (except for a slight difference in the actual length of the vessels but not the length of the cord) to obtain comparable data.

Of fundamental importance for determination of these transfer rates was the existence of a steady state within this system during the period over which measurements were made. By purely mechanical means a steady state can not be enforced in this system unless the organ remains in its normal healthy state. Utilizing the experience of others(3,9) only Group O, Rh negative blood was used as perfusion fluid, to prevent edema of the cord and transient or progressive changes in the size of the vessels. Under constant oxygen and carbon dioxide tension such perfusions can be carried on indefinitely(9).

The present preparation falls short of a direct *in utero* analogy in several respects. These are probably of quantitative rather than qualitative significance. Arterial and venous blood passing through the cord should differ in oxygen and carbon dioxide content. Perfusion rate should be more than twice that achieved in the present experiments, and finally, the theoretic interpretation does not take into account the fact that the effective size of the fetal compartment must be smaller than the volume of blood actually used.

In these preliminary experiments the primary objective was to keep the conditions constant and to demonstrate the existence of an exchange. More refined studies will clarify the relation to other factors.

*Summary.* 1. Fresh human umbilical cords 20 cm in length were perfused with heparinized Group O blood while immersed in a solution the composition of which was similar to amniotic fluid. 2. Tritiated water was added to the perfusion fluid (blood) or the surrounding bath and the change in specific tritium activity determined as a function of time. 3. Rate of exchange of water between the 2 compartments was calculated to be 40.5 and 50.5 ml of water per hour. 4. Evidence is presented that the umbilical vessels and Wharton's jelly play a significant role in the water exchange of the amniotic fluid.

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Received March 20, 1961. P.S.E.B.M., 1961, v107.

## Hemagglutination and Hemagglutination-Inhibition with Coxsackie B Viruses. (26708)

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Coxsackie B virus infections are common occurrences in man and are responsible for several distinct syndromes, such as aseptic meningitis and pleurodynia, as well as for febrile illnesses which cannot be readily differentiated from those caused by other infectious agents. Although these viruses can usually be isolated from the throat or anal specimens of patients by the use of primate tissue cultures or suckling mice, these procedures are relatively cumbersome and are still beyond the scope of most clinical laboratories. The report that Coxsackie B type 3 virus agglutinated human erythrocytes(1), and the recent finding in this laboratory that certain strains of Coxsackie B virus types 1 and 5 also possessed this property, suggested that it might be possible to develop a hemagglutination-inhibition (HI) test which would be of use in diagnosis of human infections with these viruses. This report will describe such an HI procedure and the results obtained with it in a study of 25 children infected with Coxsackie B virus types 3, 4, and 5.

*Materials and methods.* The hemagglutinating strain of Coxsackie B virus type 3 was obtained from Dr. M. Goldfield. The hemagglutinating strains of Coxsackie B virus types 1 and 5 were found in this laboratory among a number of previously unidentified enteroviruses isolated in Toluca, Mexico and kindly made available to us by Dr. A. B. Sabin.

The materials and methods employed in hemagglutination and hemagglutination-inhibition procedures were the same as those used with reoviruses(2) unless otherwise noted. Rabbit antisera were prepared by an immunization schedule described previously (3). Neutralization tests with Coxsackie B virus type 4 were carried out in rhesus kidney cell cultures as described previously(3) using the "Powers" strain of this virus(4).

The human sera employed were from institutionalized nursery children who were under observation in a longitudinal study(5).

*Results.* The hemagglutinating properties of Coxsackie B virus types 1 and 5 were discovered during an investigation in which unidentified enteroviruses were systematically screened for their ability to agglutinate human erythrocytes. When a hemagglutinating virus could not be typed by HI as a serotype known to possess this property, additional identification technics were used until the agent was identified or was recognized as a new serotype. The identity of the type 1 and 5 strains was established by complement-fixation(6), then confirmed by HI using rabbit antisera. The strain of Coxsackie B type 3 virus described by Goldfield *et al.* is the only hemagglutinating strain of this type which we have encountered. A number of other Coxsackie B type 1 and 5 strains had been tested previously for hemagglutination with negative results.

An investigation of the conditions under which Coxsackie B virus types 1, 3, and 5 hemagglutinated revealed that optimum conditions for each of the 3 types were a sedimentation temperature of 37°C using erythrocytes of newborn infants. Blood from newborn infants was obtained from the umbilical cord when it was severed at time of delivery. If the erythrocytes were to be used in HI tests, type "O" cells were employed.

TABLE I. Hemagglutination-Inhibition Antibody Responses of 10 Children Infected with Coxsackie B Virus Type 3.

| Age, mo | H-I titer with indicated antigen | | |
|---|---|---|---|
| | B-1 | B-3 | B-5 |
| 12 | <10 | <10 | <10 |
| | <10 | ≥160 | <10 |
| 13 | <10 | <10 | <10 |
| | <10 | 20 | <10 |
| 15 | <10 | <10 | <10 |
| | <10 | 40 | <10 |
| 25 | <10 | <10 | <10 |
| | <10 | 20 | <10 |
| 27 | <10 | <10 | 20 |
| | <10 | 20 | 40 |
| 29 | <10 | <10 | <10 |
| | <10 | 40 | <10 |
| 33 | <10 | <10 | <10 |
| | <10 | 20 | <10 |
| 34 | <10 | <10 | 20 |
| | <10 | ≥160 | ≥320 |
| 34 | <10 | <10 | 10 |
| | <10 | ≥160 | 10 |
| 40 | <10 | <10 | 20 |
| | <10 | 80 | 40 |

TABLE II. Hemagglutination-Inhibition Antibody Responses of 8 Children Infected with Coxsackie B Virus Type 4.

| Age, mo | H-I titer with indicated antigen | | |
|---|---|---|---|
| | B-1 | B-3 | B-5 |
| 13 | <10 | 10 | <10 |
| | <10 | <10 | <10 |
| 17 | <10 | <10 | <10 |
| | <10 | <10 | <10 |
| 18 | <10 | <10 | 80 |
| | <10 | <10 | 20 |
| 19 | <10 | <10 | 20 |
| | <10 | <10 | 10 |
| 28 | <10 | 40 | <10 |
| | <10 | 640 | <10 |
| 31 | <10 | <10 | 160 |
| | 20 | <10 | 160 |
| 33 | 40 | <10 | 640 |
| | 320 | <10 | 320 |
| 37 | <10 | <10 | <10 |
| | <10 | <10 | <10 |

TABLE III. Hemagglutination-Inhibition Antibody Responses of 7 Children Infected with Coxsackie B Virus Type 5.

| Age, mo | H-I titer with indicated antigen | | |
|---|---|---|---|
| | B-1 | B-3 | B-5 |
| 21 | <10 | 80 | <10 |
| | <10 | 80 | 40 |
| 22 | <10 | <10 | <10 |
| | <10 | <10 | 320 |
| 22 | <10 | 80 | <10 |
| | 20 | ≥320 | 80 |
| 24 | <10 | <10 | <10 |
| | <10 | <10 | 20 |
| 24 | <10 | 80 | <10 |
| | <10 | 80 | 10 |
| 26 | <10 | <10 | <10 |
| | <10 | <10 | 80 |
| 32 | <10 | <10 | <10 |
| | <10 | <10 | 40 |

Hemagglutinin titers of 1:128 or 1:256 per 0.4 ml were obtained with each of the 3 types. Partial agglutination patterns, similar to those which have been reported with certain adenoviruses and rat erythrocytes (7), were usually seen with type 3, and sometimes with types 1 and 5, when erythrocytes from adult donors were employed. Erythrocytes from most infants gave complete patterns. Hemagglutinin titers were almost always higher with cells from infants as compared with those from adults. Some variation from one lot of cord cells to another was observed.

On the basis of the above finding an HI procedure was developed which was identical with that described for reoviruses except that erythrocytes from newborn infants were substituted for those from adults and the erythrocytes were allowed to sediment at 37°C (in a warm air incubator) instead of at room temperature.

The results obtained with this procedure in testing sera of children naturally infected with Coxsackie B virus types 3, 4, and 5 are shown in Tables I, II, and III respectively. In each instance the first serum of each pair was obtained prior to infection and the second from 2 to 15 weeks afterwards. Diagnosis had been established in each child by virus isolation and sera were not selected with respect to their antibody responses. A rise in homologous neutralizing antibody was demonstrated in each pair of sera from the children with type 4 infections.

It will be noted that with one exception a significant homologous antibody response was detected in each of the 18 children with type 3 and 5 infections and that a significant heterologous response occurred in 2 of these children as well as in 3 of the 8 children with type 4 infection. In each case where a heterologous response was observed the child had pre-existing HI antibody to one or more of the 3 test antigens.

*Discussion.* The demonstration that certain strains of Coxscakie B viruses agglutinate human erythrocytes, while others do not, should encourage the search for hemagglutinating strains of enteroviruses for which this useful property has not yet been demonstrated. Thus far, in addition to the 3 Coxsackie B viruses, we have been able to confirm the specificity of the hemagglutination previously reported(1,8,9,10,11) for only the following types of enteroviruses: ECHO types 3, 6, 7, 11, 12, 13, 19 and Coe virus. We have also been able to demonstrate specific hemagglutination by a strain of ECHO type 20 (but not the prototype), ECHO type 21, Coxsackie A types 20 and 24, and several newly recognized enteroviruses which have not been numbered. There is every reason to believe that this list will be extended.

Although HI responses observed in children infected with Coxsackie B viruses were relatively type-specific, the occurrence of heterologous responses in some children with pre-existing HI antibody suggests that this degree of specificity will not hold true with older individuals. Additional data are obviously desirable on the responses of adults, time of first appearance of HI antibodies, and duration of antibody persistence. In view of the simplicity of the HI procedure as compared with other serologic tests available, it would appear that this technic warrants further investigation as to its usefulness for diagnostic and epidemiologic purposes.

*Summary.* Agglutination of human erythrocytes by Coxsackie B virus types 1 and 5 is reported. Optimum titers were obtained with a sedimentation temperature of 37°C and the erythrocytes of newborn infants. A hemagglutination-inhibition (HI) procedure employing Coxsackie B virus types 1, 3, and 5 was used to test paired sera from 25 children infected with Coxsackie B virus types 3, 4, and 5. A significant homologous response was detected in every case except one. A few heterologous responses occurred in children with pre-existing HI antibody to one or more of the 3 test antigens.

The authors are indebted to Mrs. J. Hovis for valuable technical assistance.

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Received May 1, 1961. P.S.E.B.M., 1961, v107.

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# Induced Reactivation of Herpes Simplex Virus in Healed Rabbit Corneal Lesions.* (26709)

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The first report of induced reactivation of herpes simplex infection in an experimental animal was that of Good and Campbell(1) who subsequently reported additional observations of induced herpetic encephalitis in rabbits which included virus isolation(2,3). Recently Schmidt and Rasmussen(4) reported the apparent reactivation of herpes

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* This investigation was supported in part by grants from Nat. Inst. Health, Division of Allergy and Infect. Dis., Am. Cancer Soc., and Lillian A. Margolyes League, Inc.

TABLE I. Reactivation of Herpes Simplex Virus in Rabbit Cornea by Arthus Phenomenon.

| Rabbit No. | Date and site of infection | Antibody titer (9/7/60) | Corneal Arthus reaction | | Virus release* |
|---|---|---|---|---|---|
| | | | Date | Degree | |
| 148 | 2/18/60 (L) | 1:128 | 4/27/60 | severe | + |
| | | | 6/ 8/60 | " | 0 |
| | | | 9/ 7/60 | minimal | + |
| 150 | 4/13/60 (L) | 1:8 | 6/ 8/60 | minimal | 0 |
| | | | 12/ 7/60 | " | 0 |
| 151 | 3/21/60 (L, R) | 1:512 | 6/ 8/60 | severe | + |
| | | | 9/ 7/60 | " | + |
| | | | 12/ 7/60 | " | + |
| 152 | 3/21/60 (L, R) | 1:512 | 6/ 8/60 | severe | + |
| | | | 9/ 7/60 | minimal | 0 |
| | | | 12/ 7/60 | moderate | 0 |
| 153 | 4/13/60 (L) | 1:128 | 6/ 8/60 | minimal | 0 |
| | | | 12/ 7/60 | " | + |
| 156 | 7/15/60 (L) | 1:128 | 9/ 7/60 | moderate | 0 |
| | | | 12/ 7/60 | severe | 0 |
| 157 | 7/15/60 (L) | 1:2048 | 9/ 7/60 | severe | 0 |
| | | | 12/ 7/60 | " | 0 |
| 158 | 7/15/60 (L, R) | 1:512 | 9/ 7/60 | severe | 0 |
| | | | 12/ 7/60 | " | 0 |

L = Left eye; R = Right eye.

* Herpes simplex virus recovery by corneal swabbing during the 14-day post-Arthus period.

simplex encephalitis from the latent state in rabbits by repeated intramuscular injections of epinephrine.

These reported reactivations of herpes simplex from the latent state in animals all have the disadvantage of being at an inaccessible site. The encephalitis induced is frequently fatal and manipulations and detailed observations of these animals are technically difficult. For these reasons, methods for reactivation of healed herpes simplex infection in the skin or mucous membranes were investigated in this laboratory. Repeated attempts at reactivation of healed cutaneous herpes simplex infection in mice, rabbits, and guinea pigs were unsuccessful. The stimuli for attempted reactivation included epidermal sensitization, artificial fever, local and systemic hydrocortisone injections, ultraviolet irradiation and intravenous administration of influenza virus. Attempts at reactivation of healed herpes simplex in the rabbit cornea by ultraviolet irradiation were unsuccessful. The experiments reported here have to do with attempts at reactivation of healed rabbit corneal herpes simplex infection by induction and evocation of local hypersensitivity reactions of the Arthus type.

*Materials and methods.* The strain of herpes simplex virus used was isolated in chick embryo fibroblast cell culture in this laboratory in 1957 from a labial herpes simplex lesion. A suspension of this virus was prepared in rabbit kidney cell monolayer cultures. This preparation had a titer of $10^{7.5}$ $\mathrm{TCD}_{50}$ on rabbit kidney cell culture and was used for initiation of infection and for antibody titrations.

Male albino rabbits of 3-8 lb weight obtained from Blue Spruce Farm, Altamont, N. Y. were used throughout these experiments.

Neutralizing antibody titers are expressed as that dilution of serum neutralizing 100 $\mathrm{TCD}_{50}$ of herpes simplex virus on rabbit kidney cell cultures.

Rabbit kidney cell monolayers were employed for isolation and titration of virus and for neutralizing antibody determinations, using appearance of cytopathic effect as an indicator. The maintenance medium for these cultures was 2% calf serum in 199.

*Results.* (Table I). Five rabbits were infected on the cornea without scarification by dropping approximately 0.1-0.2 ml of undiluted virus suspension ($10^{6.5}$ $\mathrm{TCD}_{50}$) into the eye and holding the eyelids closed and in firm contact with the globe for about 30 seconds. Three animals were infected unilaterally and 2 bilaterally. An additional group of 3 rabbits was similarly infected, 2 unilaterally and one bilaterally, but the cornea was scarified with a hypodermic needle prior to introduction of the virus.

Within a period of 2-4 days muco-purulent exudate, chemosis and photophobia were noted in the inoculated eyes. Rabbit kidney cell cultures inoculated with the exudate exhibited cytopathic effect similar to that seen with herpes simplex virus. The cornea showed areas of staining when a 2% fluorescein solution was applied indicating damage to corneal epithelium. The acute inflammation subsided and epithelial repair was completed within approximately 14 days, frequently leaving residual corneal scarring and vascularization. Herpes simplex neutralizing antibody was demonstrable in each rabbit so infected. Of 8 rabbits 3 had titers of 1:512 and 3 titers of 1:128; the 2 remaining animals had titers of 1:2048 and 1:8, respectively.

Beginning 6 to 9 weeks after initiation of the corneal infection, the rabbits were prepared for a corneal Arthus reaction by a modification of a technic described by Rich (5). Each animal received 0.5 ml of horse serum subcutaneously and 0.1 ml intradermally at 3-5 day intervals until an area of hemorrhage and edema appeared at site of injection. These lesions sometimes proceeded to eschar formation and ulceration. The desired reaction was obtained after 4-5 injections.

Both eyes of each sensitized rabbit were first swabbed for the presence of virus, then a small bleb was raised with horse serum at the site of corneal scarring in the left eye of each animal. Within 24 hours, varying degrees of reaction were noted, varying from slight corneal opacity to hemorrhage and in some instances ulceration and sloughing of corneal tissue. The acute corneal reaction usually subsided in 7-10 days. Several instances of deep ciliary flush were noted. Degree of reaction closely paralleled amount of corneal vascularization present at time of challenge.

Following the challenge injection of horse serum into the cornea swabbing for virus was done, usually at daily intervals, for a 2 week period. The uninjected right eye of each rabbit was also cultured for virus. (Three rabbits had healed herpes simplex infections of the right eye and 5 had had no infection of the right eye—Table I.) Induction of the Arthus reaction was repeated for a total of 2-3 times at intervals of 2½ to 6 months for each animal. In addition control cultures were taken daily to 3 times weekly for a period of 36 days on 5 rabbits, followed by weekly to twice weekly swabbings and cultures for an additional 6 months interrupted by one to 2 additional attempts to induce reactivation. Positive virus cultures from the challenged left eye during the 14 day period following an Arthus reaction were regarded as evidence of induced reactivation. All other positive cultures from either eye were interpreted as reflecting spontaneous reactivation.

The results of these studies are graphically illustrated in Fig. 1. Rabbits 156, 157 and 158 are omitted from consideration here because virus isolation attempts were entirely negative in these animals. It is of interest that these 3 rabbits had been infected using a corneal scarifying technic while the remaining 5 rabbits were infected without scarification.

One rabbit (151) had induced reactivations on 3 occasions, one (148) on 2 occasions, and 2 (152 and 153) each had one induced reactivation. In summary, of 19 attempts to induce reactivation, 7 were followed by virus release within a period of 1-12 days. No correlation was noted between degree of hypersensitivity reaction and incidence of virus excretion provided at least a minimal reaction was obtained.

When virus release occurred after attempted induction, it was demonstrable for 3-7 days except for one instance in rabbit 148 where it was detected on only one day. Virus recovery during control study periods was usually for a period of one day. It is possible that these periods would have been

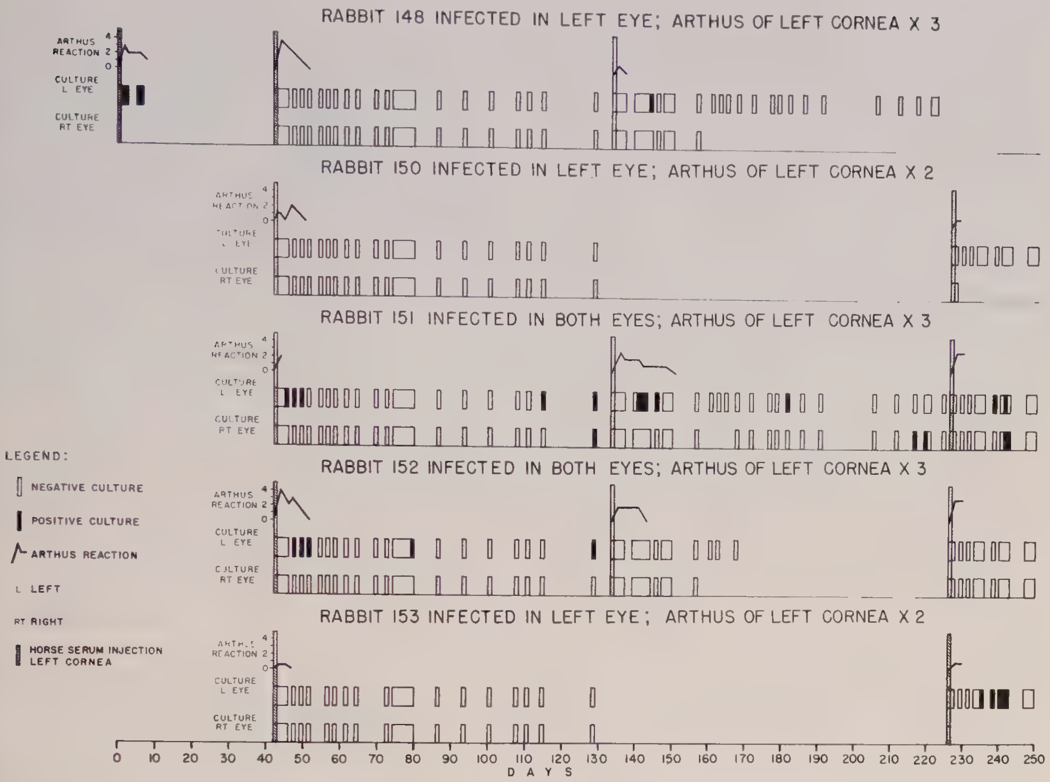

FIG. 1. Reactivation of herpes simplex virus in rabbit cornea by Arthus reaction.

longer had daily specimens been taken. There were no instances of recovery of virus from a previously uninfected eye.

It was noted that in rabbits 148, 151 and 152 where multiple induced reactivations occurred, the responses were noted at longer intervals after each challenge.

Spontaneous virus release (unrelated to injection of horse serum) occurred in only 2 of 8 rabbits (151 and 152). These 2 and 2 others (148 and 153) also released virus after Arthus reactions were induced. Neither spontaneous nor Arthus-related virus release was noted at any time in the 4 remaining rabbits.

Three rabbits (151, 152 and 158) had healed bilateral infections. Neither induced nor spontaneous virus release was detected in rabbit 158. Virus was recovered in each of 3 attempts at Arthus-induced release from the left eye of rabbit 151. Spontaneous release of virus was noted on 6 occasions in 119 control cultures from rabbit 151. These 6 positive cultures were equally divided between the 2 eyes. Rabbit 152 released virus once from the challenged left eye after Arthus reaction. Two other attempts at reactivation were unsuccessful. Spontaneous virus release was noted twice in 85 control cultures from this rabbit. This spontaneous shedding involved the left eye, in which Arthus-induced virus release had occurred.

*Discussion.* Within an arbitrarily defined period of 14 days during which time the effects of the Arthus reaction were presumably active, there were 7 instances of virus release following 19 attempts at induction. It is probably significant that during this same period virus release was not detected in the unchallenged corneas of rabbits in which bilateral infection had been induced.

The occurrence of spontaneous reactivations makes it desirable to extend these studies before one can state definitely that local anaphylaxis induces herpes simplex virus reactivation in healed rabbit corneal lesions. It seems probable that the isolation of herpes simplex virus from 2 of 28 excised post-infection rabbit corneas reported by Sery(6) may have coincided with similar spontaneous reactivation of infection—perhaps immediately prior to excision.

The repeated isolation of herpes simplex virus with and without attempts at induction and interspersed by long periods of absence of virus release is of considerable interest. Corneal herpes simplex in the rabbit thus closely parallels the syndrome of recurrent human cutaneous, ocular and mucous membrane herpes simplex infection and affords an accessible model for experimental study.

*Summary.* Herpes simplex virus was inoculated into corneas of 8 rabbits with the induction of herpetic keratitis which proceeded to healing. The animals were then sensitized to horse serum and an Arthus reaction was induced in the healed cornea. Herpes simplex virus was repeatedly recovered in rabbit kidney cell culture from swabbings taken from these corneas. This phenomenon was observed 7 times in 19 attempts at induction. While there were several instances of spontaneous virus release, none occurred during the 14 day post-Arthus period in the unchallenged eyes of animals in which bilateral infection had been induced previously.

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Received May 4, 1961. P.S.E.B.M., 1961, v107.

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## Beta-2A (=Beta-3-II=Gamma-1A) Mouse Leukemia with "Flame Cells" in Leukemic Infiltrations and Degenerative Lesions in Muscles.* (26710)

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Gamma globulin-like paraproteins have been described in various mouse transplantation lines characterized by plasmacellular leukemic(1-6) or myelomatous (7-10) lesions. More recently a transplantation line associated with macroglobulinemia and with microscopic lesions comparable to those seen in Waldenström's macroglobulinemia in man was described(11). This report concerns the new observation that the paraprotein of a transplantation line of leukemia appeared immunoelectrophoretically to be of a type homologous to the recently described human beta-2A (=gamma–1A) myeloma proteins (12-13). In addition, some of the cells ("flame cells") present in the leukemic infiltrations resembled those very recently considered to be pathognomonic of human beta-2A myelomas(14). Degenerative lesions in cardiac and skeletal muscles similar to those described in thiamine-deficient mice(15) were also observed.

*Material and methods.* Methods of paper- and immunoelectrophoresis etc. have been described(5,16,17). For estimation of hexose content of paraproteins, total protein-bound hexose (anthrone method) was related to scannings of PAS-stained paper electrophoreses of whole sera(18). Ultracentrifugation was performed at 60,000 rpm in an oil-driven ultracentrifuge (Svedberg type) on serum diluted 4 times in phosphate buffer, pH 6.66, containing 0.2 M sodium chloride. The *transplantation line* originated in a 28-months old, untreated (CBA × DBA/2) $F_1$ mouse(19) with enlarged lymph nodes and

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* Supported by grants from Danish State Research Foundation, and Danish Rheumatism Assn.

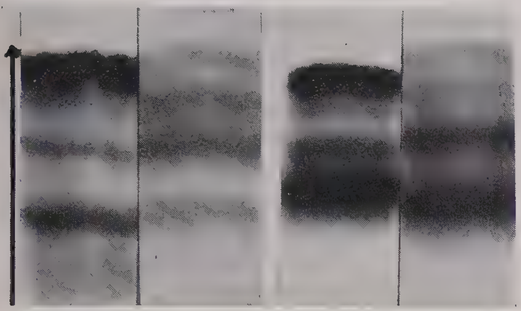

FIG. 1. Bromphenol blue-stained and PAS-stained electrophoreses of normal serum (left) and pathological serum (right); in the latter the heavily PAS-positive beta-fraction constituted 45% of total protein, in the former, 19%. Bromphenol blue-stained strips were run on 10 μl, PAS-stained on 20 μl of serum.

renal amyloidosis. Following subcutaneous inoculation of leukemic tissue, percentage of takes through 10 transfers was 80-100, survival time 9-10 months, gradually shortening to 2 to 3 months.

*Results.* A. *Serum proteins.* a) Total serum protein was found normal in earlier passages, then gradually decreased to about 4 g%. b) *Paper electrophoresis* (Fig. 1) revealed an increased beta globulin fraction, which in some mice, particularly of the earlier passages, appeared as 2 more or less merging bands. This fraction was highly PAS-positive (Fig. 1). c) *Hexose content* of the paraprotein from a mouse of the sixth passage was found to be 6.7%. Corresponding values for the gamma-type mouse paraproteins(4-6) ranged from 1.0 to 2.8%. d) *Immunoelectrophoretical analysis* of the pathological serum (Fig. 2A) showed the paraprotein as a heavy bow lying in the beta-2 area and apparently being (a) part of the gamma-globulin line. Evidence for some heterogeneity was visible from a deflection of the abnormally extended anodal end of the paraprotein bow. In addition a dense precipitate in the vicinity of the filling hole appeared to correspond to some amount of serum albumin migration with beta velocity. The anodal end of this precipitate could be followed into the cathodal end of the normal serum albumin bow. Both disappeared after absorption of the anti-mouse antiserum with purified mouse serum albumin, thus demonstrating their identity.

By using an antiserum produced against the microsome fraction of the leukemic tissue, a clearer and more intense picture of the serum paraprotein could be obtained (Fig. 2B). Confrontation of such a pattern with the one of normal white mouse serum, using the technic of interrupted slits(13,17), showed the paraprotein bow to fuse intimately with a normal bow previously described as beta-3-II(16). Recent studies on normal white mouse serum have indicated identity between this beta-3-II globulin and the protein of human serum known as beta-2A-globulin (=gamma–1A–globulin). The paraprotein also showed evidence for cross-reaction with the normal gamma-globulin (Fig. 2B). The same relationships between paraprotein on one hand and normal beta-2A and gamma-globulin on the other hand were also evident from an experiment (Fig. 2C) in which the antiserum against the microsome fraction was used for confrontation of a normal white mouse serum and a mixture of normal and pathological serum. The superimposed paraprotein bow here obviously cross-reacted both with normal beta-2A and normal gamma-globulin. The free cathodal tip of the paraprotein bow is to be interpreted as evidence for the existence of pathologic, antigenic determinants, whose corresponding antibodies were present in the homologous antiserum here employed. e) *Ultracentrifugation* of serum from one mouse (Fig. 3) revealed the following components: S 4.2 77%, S 6.2 10%, S 11.6 8%, S 16.8 5% (normal values: S 4.5 89%, S 7.8 2%, S 17.9 9%), thus demonstrating an excess of the S 6-8 component and 8% of a S 11.6 component. In serum from another mouse similar findings were observed.

B. *Macroscopic lesions* were marked enlargement of all lymph nodes, thymus, spleen and liver, but no growth at site of inoculation In some mice with severe leukemic infiltration, fine, sharp, white streaks were seen in the cardiac and lumbar musculature.

C. *Microscopic lesions* were infiltration of typical leukemic localization in the above mentioned organs, increasing in severity through the serial transfers. The cells of the infiltrations were of 2 types (Fig. 4A): a)

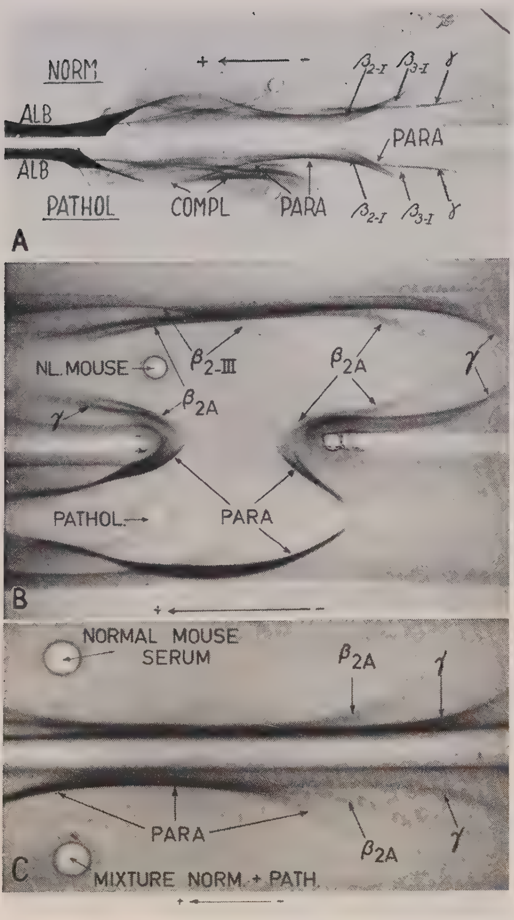

FIG. 2. Immunoelectrophoreses. A. Normal and pathological serum developed with anti-normal mouse serum antiserum. The lower pattern shows the heavy paraprotein line cathodally ending in the remnant of the normal gamma bow and anodally extending into the slow alpha-2 area, where the albumin-paraprotein complex is also visible. B. Interrupted slit technic using antiserum against homologous tumor microsome fraction and confronting the patterns of pathological (lower half) and normal white mouse serum (upper half). Fusion of paraprotein bow with both normal beta-2A-globulin and normal gamma-globulin is evident. C. Confrontation of normal white mouse serum alone (upper part) and a mixture of the latter with pathological serum (lower part), using anti-microsome fraction antiserum. Cross-reaction is obtained between paraprotein and both normal beta-2A- and normal gamma-globulin. The free cathodal tip of paraprotein corresponds to pathological antigenic determinants reacting with their homologous antibodies.

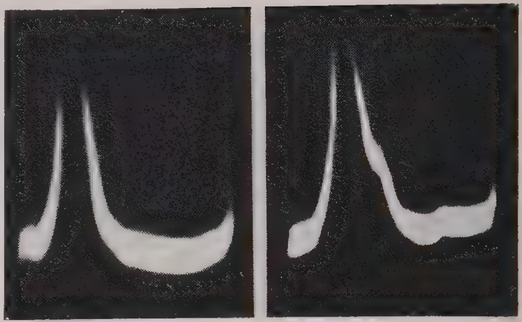

FIG. 3. Ultracentrifugation diagrams; exposure after 50 min. A. Normal mouse serum. B. Pathologic serum showing S 6 and S 11 components.

rather small, closely packed cells with scanty, slightly basophilic cytoplasm and relatively large, round, ova or angular nuclei with a strong nuclear membrane and with a low chromatin content, arranged in small clumps connected by fine threads, and b) much larger cells with sharply demarcated cell borders and with very abundant eosinophilic or neutrophilic cytoplasm, moderately positive to the PAS-reaction, occasionally of a glassy appearance and sometimes showing minute vacuoles; the nuclei were small, round, rather dark and definitely eccentrically located. In Giemsa-stained imprints (Fig. 4B) the first-mentioned cell type appeared as cells with large, round, intensely staining nuclei with delicate chromatin pattern and scarce, moderately basophilic cytoplasm. The other cell type was seen as light cells of varying size characterized by very small, dense, eccentrically placed nuclei, and abundant, inhomogeneous basophilic cytoplasm; in a varying number of these cells, however, the cytoplasm, except for a juxtanuclear space, was filled with minute eosinophilic spots, which in the peripheral part of the cytoplasm coalesced to smaller or larger eosinophilic areas (so-called "flame cells"). Numerous naked nuclei were always seen in the imprints.

In the latest passages the large, light cells were seen diffusely spread as single cells among the cells of the small type; they were most numerous in the lymph nodes, where they occurred in large numbers in the severest cases accumulating to whole coherent areas; they were less numerous in the spleen and more rarely seen in the perivascular liver infiltrations. After administration of cortisone to one mouse the number of these cells was found reduced. In the 5th to 7th transfers the large, light cells were located as small clusters or streaks between the small cells, preferably near the nodal sinuses or where "free space" probably had been available; they were not present in all lymph nodes of each mouse. In the very earliest passages and in the spontaneous case only a few of the light cells were present in the leukemic infiltrations.

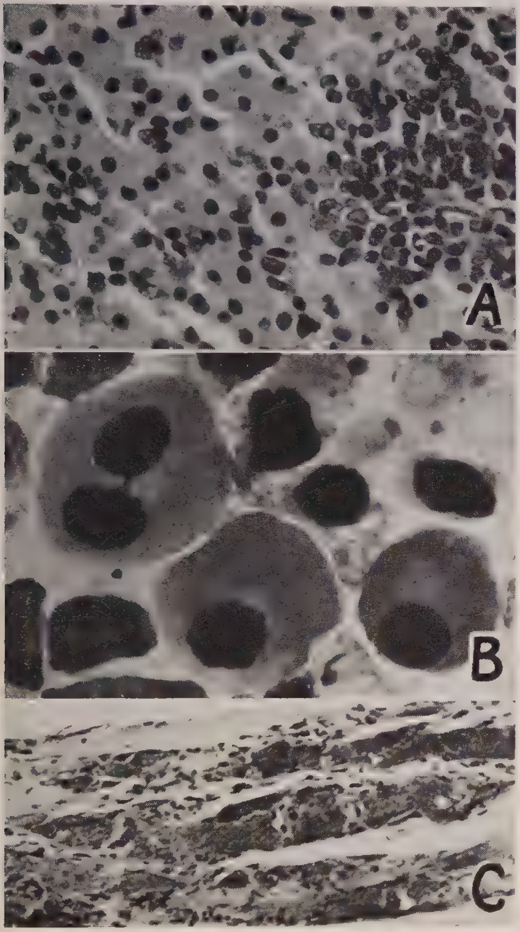

FIG. 4. A. Section of tumor infiltrated lymph node. Areas of large, polygoneal cells with abundant slightly eosinophilic or neutrophilic cytoplasm and with small dark nuclei intermingled with masses of small dark closely packed tumor cells with a rather large, round nucleus and with scarce, slightly basophilic cytoplasm with hardly visible cell borders. Hem.-Eos. × 210. B. Imprint preparation of tumor infiltrated lymph node. Three cells corresponding to the larger polygoneal cells in A; one of them binucleate. These cells are large with dense, ovoid, eccentrically located nuclei and abundant inhomogeneous cytoplasm with a clear perinuclear zone; eosinophilic areas of varying extent occupy part of the otherwise basophilic cytoplasm. The small cells with large dark nuclei and with scarce or damaged cytoplasm correspond to the small dark cells of A. Giemsa stain × 1400. C. Amorphous masses in striated muscle apparently replacing muscular fibers. The granular and hyaline appearance is visualized. Hem.-Eos. × 350.

In mice of the second transfer severe splenic amyloidosis of secondary type and in 2 mice heavy intestinal amyloidosis was observed.

The white streaks in the cardiac and lumbar muscles seen in mice with marked infiltrations appeared (Fig. 4C) as amorphous, dark staining masses often of hyaline, sometimes of granular appearance. They stained heavily with the PAS-reaction, not at all with methyl violet, only slightly with alcian blue and in the Hale reaction, and not with chromotrope 2R. Bacteria or fungi could not be detected. Moderate to severe calcification appeared in about half the cases. With few exceptions no tumor cells and no signs of inflammation were found in relation to the deposits. The same changes, although not macroscopically visible, were seen in most mice of the later passages, located as mentioned above and particularly in muscles around the larger joints.

*Discussion.* The following factors point to the mouse leukemia here reported as homologous to the beta-2A-type of human myeloma. 1) The immunological correspondence of the paraprotein with beta-2A-globulin and its cross-reaction with normal gamma-globulin, which are characteristic of human beta-2A-type paraproteins, were also found here. 2) As in many human beta-2A-myeloma cases, a slow moving albumin component probably corresponding to complex formation with the paraprotein(13) was also evident in this leukemic mouse serum. 3) The hexose content of the mouse paraprotein here described was considerably higher than in other mouse leukemias of the gamma-type, a situation corresponding to the findings in the homologous 2 human myeloma types(13). 4) The electrophoretic mobility of the mouse paraprotein here reported was of the order of magnitude of beta- and to some extent even of slow alpha-2-globulins. 5) The paraprotein appeared to be inhomogeneous in the ultracentrifuge and to contain components sedimenting with coefficients larger than 7S, as also observed in human beta-2A-type myelomas(20). 6) The peculiar type of large light cells was consistently found to be associated with the leukemia described here, in contrast with the cytological findings in ordinary gamma-type mouse paraproteinemias. Similar cells have been found to be pathognomonic of human beta-2A-myelomas (14). The degenerative lesions in cardiac and skeletal muscles, so far of unknown etiology, have not been observed in other types of mouse leukemia.

*Summary.* A transplantable mouse leukemia with the following characteristics is described: Production of a paraprotein of beta-2A-type. Presence of peculiar large light cells ("flame cells") in the leukemic infiltrations and degenerative lesions in cardiac and skeletal muscles.

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Received May 8, 1961. P.S.E.B.M., 1961, v107.

## Heterologous Transplantation of Two Tissue Culture Lines of Glioblastoma Multiforme (TC 178 and TC 224).* (26711)

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It is extremely difficult to distinguish between "normal" and neoplastic cells growing in monolayer tissue cultures on a morphological basis(2,5). Furthermore, the histological features of neoplastic tissue culture cells do not give accurate information about the tumor of origin. It was thought that heterologous transplantation of tissue culture cells might solve these problems. The present communication deals with the successful heterologous transplantation of 2 permanent lines of human glioblastoma multiforme cultured in this laboratory. Glioma TC 178 has been growing since Sept. 4, 1957, and has been reported in detail in this journal(3). The line TC 224 has been in continuous cultivation since Feb. 28, 1959. A

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* Supported by U.S.P.H.S. grant.

method of heterologous transplantation was utilized which is described in detail elsewhere (4).

*Materials and methods. A. Tissue culture procedure.* TC 178 and TC 224 were isolated from 2 different cases of human glioblastoma multiforme. The method of culture was the same in both instances. Fresh tumor tissue was minced with uterine scissors and washed several times with balanced salt solution. Small quantities of the mince were placed into several 8 ounce wide-mouthed Blake bottles. Twenty cc of culture medium (composed of Eagle's medium, glutamine† 1%, penicillin 100 units/cc, and streptomycin 100 mg/cc), containing 10% horse serum† were added to each bottle. The bottles were placed in a 37°C incubator. Growth was observed around the explants by the second day, and after 3 to 4 weeks all bottles showed a luxuriant growth. At this time the original medium and the floating necrotic debris were discarded and fresh medium was added. When the surface of the bottles was completely covered with cells, these were scraped with a rubber policeman, centrifuged at 500 rpm for approximately 10 minutes, resuspended in fresh medium and planted in new bottles. At present the medium of the cultures is changed at weekly intervals and new bottles are implanted with tissue culture cells at the same time.

*B. Transplantation procedure.* 0.03 ml and 0.1 ml of a suspension of 5 $\times$ $10^6$ cells/ml of TC 178 or TC 224 were injected intracerebrally into untreated mice and guinea pigs respectively. Approximately 5 $\times$ $10^6$ cells of glioma TC 178 or glioma TC 224 in 0.5 ml volumes were injected into the right flank of hamsters which had received, prior to inoculation, 200 r of total body radiation. The animals also received 4.5 mg of cortisone acetate on alternate days for 3 doses. Eight to 15 days after inoculation with TC cells, the animals developed subcutaneous tumors of varying size up to 1 cm in greatest dimension. The growing tumors were removed sterilely from the hamsters; parts were taken for histological examination and the rest cut into pieces approximately 3-4 mm in diameter. These fragments were transplanted into the anterior chamber of the eye or into the brain of healthy untreated guinea pigs.

*Results.* When the glioma tissue culture lines (TC 178 or TC 224) were injected into the brains of untreated guinea pigs and mice, no growth resulted.

In contrast, 3 types of lesions were found in the subcutaneous tissue of treated hamsters.

1. Small aggregates of injected cells, the majority of which were necrotic; there was no evidence to suggest that growth of these had occurred after transfer.

2. Nodules ranging up to 7 mm in diameter were found in the majority of animals. These were composed of a necrotic center surrounded by tumor cells growing in clusters without apparent organization.

3. In rare instances larger growths up to 1 cm in diameter were found; unlike the other lesions obtained, these lesions were vascularized and obviously represented true neoplasms of the transplanted cells. It was of particular interest that these tumors had an epithelial-like appearance (Fig. 1) and did not bear any morphological resemblance to human glioblastomas. To supply adequate material for investigation, a further attempt was made to transplant these interesting neoplasms to guinea pigs.

The anterior chambers and brains of untreated guinea pigs were again used as transplantation sites for growing tumor transplants from the treated hamsters. In contrast to the original transfers from tissue culture, these transplants survived and grew. Growth was rapid and within 1½ months large neoplasms were present in both brains and eyes (Fig. 2). The tumors were readily transferred to other untreated guinea pigs and have been carried by serial passage to the present time.

The histological appearance was maintained and became more apparent in the guinea pig transplants. The cells were unquestionably epithelial in character and the tumor appeared to be a carcinoma rather

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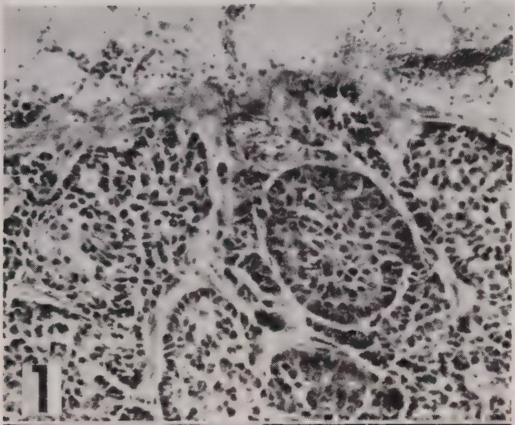
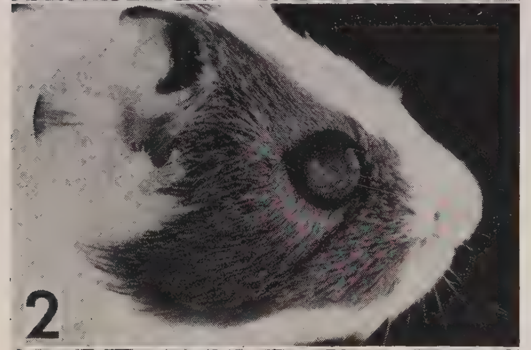
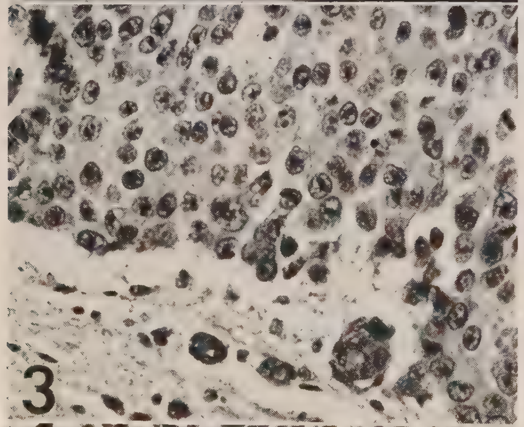
† Available from Microbiological Associates, Washington, D. C.







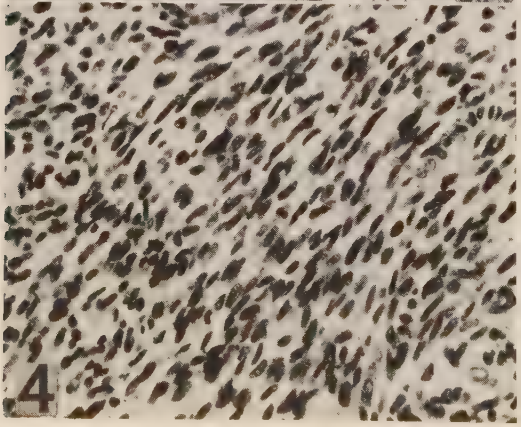


FIG. 1. Epithelial-like tumor produced by glioma TC 224 cells growing in subcut. tissue of treated hamsters. Hematoxylin and eosin stain. × 264.

FIG. 2. Well vascularized tumor produced by glioma TC 178 cells occupying anterior chamber of eye of untreated guinea pigs.

FIG. 3. Epithelial tumor produced by glioma TC 178 cells invading brain tissue of unconditioned guinea pig. Tumor has a carcinoma-like appearance. Hematoxylin and eosin stain. × 660.

FIG. 4. Tumor produced after transplantation of human glioblastoma multiforme directly into brain of untreated guinea pig. Tumor is composed of spongioblast-like cells and closely resembles the human neoplasm. Hematoxylin and eosin stain. × 660.

than a glioblastoma (Fig. 3). It is probably significant that the same morphological alteration occurred in transplants of both of the tissue culture lines (TC 178 and TC 224). It has been shown previously(1) that direct transfer of human glioblastoma tissue to the guinea pig without intermediate tissue culture and hamster transfer results in growths of typical glioblastomatous character (Fig. 4).

It is apparent that the morphological alteration occurred in the tissue culture because it was observed both in treated hamsters and untreated guinea pigs. On the other hand, the attainment of the property of heterotransplantability was somehow related to residence in treated hamsters because cells obtained directly from tissue culture were not heterotransplantable.

*Summary.* Two tissue culture lines of human glioblastoma multiforme (TC 178 and TC 224) growing in continuous cultivation have been successfully transplanted into the anterior chamber of the eye and into the brain of untreated guinea pigs after a preliminary passage through X-rayed hamsters treated with cortisone. Once heterotransplantability is established, the produced tumors can be transferred from untreated host to untreated host. Histologically the tumors are epithelial-like rather than glioblastomatous.

The author is greatly indebted to Mr. George L. Morann, Miss Phyllis E. Johnson, and Miss Betty Mullaly for technical help.

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Received May 9, 1961. P.S.E.B.M., 1961, v107.

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## Inhibition of the Infectivity of Poliovirus Ribonucleic Acid by an Interferon.* (26712)

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The site at which interferon inhibits virus multiplication has been assumed to be an intracellular one, since interferon does not interfere with adsorption or release of virus (1,2). It was thought that further evidence with regard to its site of action could be obtained using infectious ribonucleic acid (RNA). If the infectivity of intact virus but not that of infectious RNA were inhibited in a cell treated with interferon, it could be assumed that interferon acted at the site of penetration of intact virus, or at the site of reactions involved in dissociation or uncoupling of the intact virus into its infectious nucleic acid and protein components. On the other hand, if the infectivity of RNA as well as intact virus were blocked in interferon treated cells, this would be supportive evidence that the site of interference is at some point of the viral reproductive cycle mediated by infectious RNA. If in addition it could be shown that infectious RNA is adequately taken up and is not preferentially inactivated by cells treated with interferon, the thesis that interferon acts at some intracellular site of virus synthesis or assembly would be further supported. These hypotheses were tested in experiments described in the following report.

*Materials and methods. Viruses, cultures and media.* Sindbis virus (Egypt AR 339), vesicular stomatitis virus (VSV, Indiana), and poliovirus type III (Saukett) were employed. Primary cell cultures of chick embryo, monkey kidney and human amnion were prepared in 3 oz. bottles. These were nourished in medium consisting of 0.5% lactalbumin hydrolysate (LAH) in Hanks' balanced salt solution (BSS), 4% calf serum and antibiotics. Plaquing medium included in addition 1.5% bacto agar, and 1.0% or 1.5% of 1:1000 neutral red depending on whether monkey kidney or chick cultures were overlaid. *RNA.* Infectious poliovirus RNA was extracted by the cold phenol method from partially concentrated poliovirus harvests of infected Roux bottle cultures of monkey kidney or human amnion cells by the modified method of Holland *et al.*(3). The infectivity of RNA was also determined by their method(4) as follows: RNA was diluted 1:1 in 2M $MgSO_4$ and 0.2 ml was inoculated in monkey kidney cultures that had been washed sequentially with 5 ml phosphate buffered saline (PBS) and 1 ml 2M $MgSO_4$. After 15′ adsorption at room temperature, the inoculated cultures were decanted, washed with 5 ml medium and overlaid with plaquing medium. Various lots of RNA titered $10^2$-$10^3$ p.f.u./0.1 ml. Its infectivity was destroyed by incubation for 20′ at 25° with $10^{-2}$ $\mu$g/1.0 ml of ribonuclease (Worthington), or for 5′ at 25° with medium. *Interferon and controls.* An interferon, hereafter abbreviated S-VIF, was prepared as follows: Stock Sindbis virus titering $10^6$-$10^8$ p.f.u./0.1 ml was first inactivated at 56°C for 4 hours. Five-tenths ml of the inactivated material was added to a chick culture and incubated at 36° for 18 hours, after which it was decanted and washed with 5 ml of Hanks' BSS before addition of 5 ml of medium. The culture then received 0.1

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[*] Supported by Nat. Inst. Health grant. Portions of this paper were presented to Am. Soc. for Clin. Invest. May 1, 1961, Atlantic City.

ml containing about $10^7$ p.f.u. infectious Sindbis virus. On the day after infection, the fluid was collected, centrifuged at 2,500 r.p.m. and inactivated at 56°C for 4 hours before use. Control fluids (CF) were prepared concurrently with S-VIF by omitting the addition of virus. *Measures of inhibition.* When a culture was "treated" with S-VIF, 0.25 ml of undiluted S-VIF or a dilution of S-VIF made up in medium was overlaid on decanted chick cultures and incubated at 36°C for 3 hours. These cultures were then washed with 5 ml medium before use. To measure the inhibition of intact virus, treated cultures were challenged with 100-200 p.f.u. VSV. Controls that had been treated with CF were challenged concurrently. Plaques were counted on the 2nd and 3rd days after inoculation, and inhibition was expressed as a comparison of plaque counts in treated and control cultures.

The measurements of inhibition of infectivity of polio-RNA were based on a comparison of 2 parameters: 1) virus production, 2) cell infection as expressed by infectious center formations. 1) To compare virus production, S-VIF treated and control chick cultures were inoculated with RNA, replenished with 5 ml of medium and incubated for 18 to 24 hours. To titer extracellular virus, 0.1 ml aliquots of the overlay were taken at various intervals after infection and inoculated in monkey kidney cultures for poliovirus plaque formation. Total virus was similarly measured after release of intracellular virus by 3 cycles of freezing and thawing. 2) To compare infectious center formations, treated and control cultures inoculated with RNA were further washed sequentially with 10 ml LAH and 10 ml PBS at room temperature. The cells were detached from the glass surface by incubation at 36° for 5′ after addition of 1.0 ml of 0.05% trypsin and 0.01% versene to each culture. To stop tryptic activity, 4.0 ml cold medium was added and cells were sedimented by centrifugation at 800 rpm for 5′. These were washed in 5 ml medium and again centrifuged. The resulting supernatant in preliminary experiments had no detectable infective activity presumably because of the inactivating effect of medium on infectious RNA. The final cell sediment was resuspended in 2.0 ml and serial 10-fold dilutions were made in medium. Cell counts varied from $10^6$ to 6 × $10^6$ per culture. Dilutions of cells in 0.2 ml were then added to duplicate monkey kidney cultures. After incubating for one hour at 36° to allow for cell attachment, 0.5 ml of plaque overlay was first added to the monolayer to minimize cell migration before addition of the usual 7.0 ml. The number of plaques representing infectious centers were counted in 2-3 days.

*Results. Properties of S-VIF.* The multiplication of intact viruses such as VSV is inhibited in chick cultures treated with S-VIF(5). To demonstrate that the effect of S-VIF conforms to that of previously described types of interferon, it was desirable to show that the inhibitory factor is a protein like substance which is not identifiable with Sindbis virus and that it does not act by directly inactivating virus.

Experiments measuring its inhibitory activity after high speed centrifugation, treatment with antiserum to Sindbis virus and dialysis are summarized in Table I. It is apparent that the inhibitory factor in S-VIF is not substantially reduced by any of these procedures.

To test whether S-VIF directly inactivated VSV virus: 0.4 ml of a $10^{-4}$ stock solution of VSV virus was added to each of 2 separate tubes containing 0.4 ml of undiluted S-VIF and 0.4 ml of CF. The mixtures were incubated at room temperature for one hour, and titrated by plaque formation in quadruplicate chick cultures. The means of plaque counts were 51 and 52 respectively. It appears then that S-VIF does not directly inactivate VSV under these circumstances.

To test whether the inhibitory activity of S-VIF could be destroyed by a proteolytic enzyme(1), trypsin was added to S-VIF to a concentration of 0.05% and incubated at 37°. Before testing the digest for inhibitory activity, the tryptic action was stopped by adding soy-bean trypsin inhibitor (SBI) to a concentration of 0.25%. SBI was also added to control fluids (CF) in the same concentrations. Two 0.3 ml aliquots of each

TABLE I. Effect of Centrifugation, Antiserum and Dialysis on Viral Inhibitory Effect of S-VIF against VSV.

| Dilution of inhibitor (0.25 ml) | S-VIF | | S-VIF centrifuged† | | S-VIF | |
|---|---|---|---|---|---|---|
| | Not centrifuged | Centrifuged* | No antiserum | Antiserum | Not dialyzed | Dialyzed‡ |
| 1:1 | 35 | 34 | 52 | 35 | 5 | 9 |
| 1:2 | 44 | 45 | ND | ND | ND | ND |
| 1:4 | 82 | 65 | 86 | 91 | 41 | 37 |
| 1:8 | 112 | 86 | ND | ND | ND | ND |
| 1:16 | 116 | 130 | 118 | 116 | 68 | 77 |
| 1:32 | 121 | 138 | ND | ND | ND | ND |
| 1:64 | 116 | 131 | 125 | 143 | ND | ND |
| Control | 168 | | 165 | | 117 | |

Figures are the means of quadruplicate plaque counts except for those in first 2 columns, which represent duplicates.

ND = Not done.

* Centrifuged twice for a total of 20 hr at 104,000 g. Reduction in virus titer is usually from $10^8$ p.f.u. to about $10^2$ p.f.u./0.1 ml. Heat inactivated before use.

† Each dilution of centrifuged S-VIF contained a 1:4 dilution of either normal rabbit serum or an antiserum against Sindbis virus sufficient to eliminate all infective activity. These mixtures were preincubated for 30′ at 36° before they were added to chick cultures.

‡ Dialyzed in tap water for 18 hr, distilled water for 4 hr, and reconstituted in medium by 2 cycles of dialysis in LAH in Hanks' BSS for 3 and 18 hr at 4°C.

of the following 4 fluids: CF, CF plus SBI, undiluted S-VIF, and a 8′ tryptic digest of S-VIF were added to separate chick cultures and these were incubated for 3 hours at 36°. The cultures were then inoculated with VSV after being washed with 5 ml of medium. The mean plaque counts of the cultures treated with these 4 fluids were: CF alone: 148, CF + SBI: 173, S-VIF alone: 0, S-VIF after 8′ trypsinization: 112. These results indicate that the no inhibitory activity is apparent in S-VIF after tryptic digestion.

The biological and chemical properties mentioned above show that the viral inhibitor in S-VIF is not associated with sedimentable virus or virus antigen and that it is a non-dialyzable, heat stable substance inactivated by a proteolytic enzyme. It does not act by directly inactivating virus. These are the properties of a class of inhibitors called viral inhibitory factor (VIF) by us(1), and interferon by Isaacs and Lindemann(6).

*Effect of S-VIF on infectivity of RNA.* The inhibitory effect of S-VIF on the infectivity of polio-RNA in chick cultures was first tested by comparing virus production in treated and control cultures. Fig. 1 is a summary of 3 separate experiments measuring the growth curves of poliovirus in treated and control cultures inoculated with polio-RNA. Virus is produced in a one step fashion in both types of cultures at 7-11 hours after inoculation with no evidence of further cycles of infection. This is consistent with the findings of Holland *et al.*(3). Judging from the similarity of the extracellular and total virus titers when these were tested simultaneously, virus release into the medium is also complete at this time. The inhibition of virus production in S-VIF treated cultures is indicated by the fact that these produced only 1.7% to 0.8% of the amount of virus produced in control cultures, a degree of in-

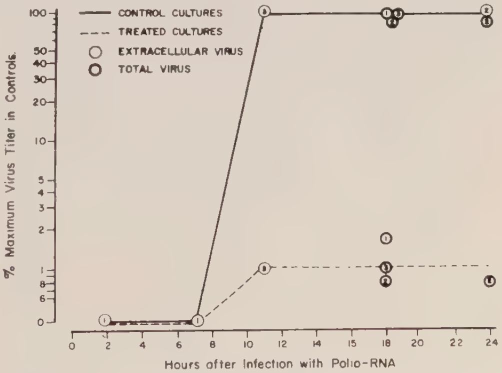

FIG. 1. Inhibition of virus production in chick cultures treated with S-VIF and inoculated with polio-RNA. Cultures were treated with undiluted S-VIF and CF and were inoculated with polio-RNA in 3 separate experiments denoted by numbers within circles. Maximum total virus produced in the control chick cultures in these 3 experiments were $10^{3.8}$, $10^{3.8}$, and $10^{5.7}$ p.f.u. respectively.

TABLE II. Inhibition of Poliovirus Yield and Infectious Center Formation in Chick Cultures Treated with Varying Dilutions of S-VIF.

| | Virus yield, % of control | | | | | No. p.f.u. $\times 10^4$ per culture | Infectious centers, % of control | | | | | No. I.C. $\times 10^2$ per culture |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Mean | | 1 | 2 | 3 | 4 | Mean | |
| Dilution | (a) | (b) | (c) | (d) | (e) | (f) | (g) | (h) | (i) | (j) | (k) | (l) |
| C.F. | 100 | 100 | 100 | 100 | 100 | 27.0* | 100 | 100 | 100 | 100 | 100 | 28.4† |
| 1:1 ‡ | 2.2 | 0 | ND | ND | 1.1 | .3 | 1.0 | 2.2 | ND | ND | 1.6 | .5 |
| 1:2 | 3.6 | 4.0 | ND | ND | 3.8 | 1.0 | 2.1 | 1.4 | ND | ND | 1.8 | .5 |
| 1:4 | 6.2 | 9.9 | 1.0 | .8 | 4.5 | 1.2 | 3.9 | 15.0 | 4.6 | 17.0 | 10.1 | 2.9 |
| 1:8 | 8.6 | 11.0 | 4.9 | 3.9 | 7.1 | 1.9 | 15.4 | 10.0 | 20.0 | 9.6 | 13.8 | 3.9 |
| 1:16 | 71.0 | 34.0 | 2.4 | 10.0 | 29.4 | 7.9 | 21.2 | 11.0 | 42.0 | 15.7 | 22.5 | 6.4 |
| 1:32 | ND | ND | 20.2 | 55.5 | 37.9 | 10.2 | ND | ND | 37.0 | 23.0 | 30.0 | 8.5 |

1, 2, 3, 4 represent separate experiments; C.F. = control fluid; p.f.u. = plaque forming units; I.C. = infectious centers; ND = not done.

a, b, c, d: Calculated as follows: virus yield in S-VIF treated cultures ÷ virus yield in controls × 100.

e: Mean of a, b, c, d.

f: e × 27.0; * 27.0 × $10^4$ = mean total virus yield in control cultures.

g, h, i, j: Calculated as follows: No. I.C. in S-VIF treated cultures/cell count of culture ÷ No. I.C. in control culture/cell count in control × 100.

k: Mean of g, h, i, j.

l: k × 28.4; † 28.4 × $10^2$ = mean of total I.C. in control cultures.

‡ Dilution of S-VIF applied to chick cultures.

hibition at least as great as that expressed by reduced plaque formation in cultures treated with S-VIF and challenged with VSV (Table I).

To test whether or not inhibition of virus production in S-VIF treated cultures was accompanied by inhibition of cell infection as well, both virus production and infectious centers were titrated in a series of cultures treated with different concentrations of S-VIF. Duplicate chick cultures treated with CF and serial dilutions of S-VIF were inoculated with undiluted polio-RNA. One set was allowed to incubate for 18 hours for measurement of total virus yield, while the other set was used for titration of infectious centers. Four separate such experiments using one lot of S-VIF and polio-RNA are summarized in Table II. Three observations may be made from these data: 1) both infectious center formations and virus yield are inhibited in S-VIF treated cultures; 2) the magnitudes of inhibition of these 2 parameters at each dilution of S-VIF are comparable. This suggests that the inhibition of virus yield is due to prevention of cell infection, and that inhibition of virus yield is therefore an all or none phenomenon. However there is too great a fluctuation of the amount of inhibition from experiment to experiment to state this with certitude. 3) It appears that if we were to plot either inhibition of virus yield or inhibition of cell infection with varying concentrations of S-VIF, we would not obtain a linear curve. There is much less increase in inhibition at higher concentrations of S-VIF than at lower.

*Direct effect on polio-RNA.* From the foregoing it is apparent that S-VIF prevents both infection of cells and production of virus. This inhibition may be due to a block of a reaction required for virus synthesis within the cell. Alternatively, it may be postulated that either 1) RNA is inactivated at the cell surface or within the cells of cultures treated with S-VIF, or 2) adsorption of infective RNA is impaired in treated cells.

To test these possibilities, 3 types of experiments were undertaken. In the first, an attempt was made to see if inhibition of virus yield was obtained if S-VIF was added *after* inoculation of polio-RNA. Two chick cultures were infected with 0.2 ml polio-RNA suspended 1:1 in 2M $MgSO_4$. After washing, one of these received 0.3 ml undiluted S-VIF, while the other received 0.3 ml CF. The 2 cultures were incubated at 36° for 3 hours, after which the fluids were washed off and 5 ml medium added to each culture. After 18 hours of incubation at 36°, total virus titrations were carried out by making 10-fold serial dilutions of aliquots from both cultures and inoculating 0.1 ml in monkey kidney tube cultures. Titers for treated and control cultures were $10^{2.5}$ and $10^{3.5}$ $TCD_{50}$/0.1 ml respectively. The fact that inhibition of virus yield is detectable *after* adsorption of polio-RNA in treated cells suggests that at least some of the inhibition cannot be accounted for by inhibition of adsorption of RNA, or by inactivation of RNA at the cell surface of treated cells.

In the second type of experiment, residual RNA was titrated after exposure to S-VIF treated cells. 0.2 ml of a 1:1 dilution of polio-RNA was added to 2 of each of the following types of chick cultures: 1) control cultures with CF, 2) cultures treated with undiluted S-VIF and 3) blank bottles. At various time intervals, residual RNA was titrated by diluting the contents of a bottle 1:10 and titrating on duplicate monkey kidney cultures. At 0 hour, the RNA in a blank bottle titrated 70 p.f.u. representing the initial inocula; at 10′, the residual RNA in control and treated cultures titrated 39 and 41; at 30′, the corresponding values were 21 and 21. The RNA in the blank bottle also measured 21 p.f.u. at this time. Figures of similar magnitude were found in repeat experiments. It is apparent that there is no difference between rate of inactivation of RNA upon exposure to control and treated cells. On the other hand, there is also a fairly rapid rate of degradation of RNA in $MgSO_4$. This precludes accurate measurements of the adsorption of the infectious RNA in either the control or treated cultures by this method. However, in so far as the residual titers reflect adsorption, there is no difference between the control and treated cultures.

In the third type of experiment, cells were scraped from a control and a S-VIF treated culture and suspended separately in 0.5 ml 2M $MgSO_4$. After freezing and thawing the suspension 10× to release intracellular contents that may preferentially inactivate polio-RNA 0.15 ml of undiluted RNA was added to each of the suspensions and these were incubated at 37° for one hour. The initial titer was 80 p.f.u. and the mean of duplicate plaque counts for the control and treated cultures at 10′, 30′ and 60′ were respectively: 49 and 52; 32 and 42; 14 and 24. Such results indicate that under the assumptions and conditions described, there is no release of intracellular RNA inactivating material.

These 3 types of experiments taken together indicate that the inhibition of infectivity of polio-RNA in S-VIF treated cells cannot be entirely accounted for by inhibition of adsorption of polio-RNA, and no evidence for an intracellular RNA inactivating substance in S-VIF treated cells was found.

*Discussion.* The inhibition of virus production in interferon-treated cells inoculated with polio-RNA is presumptive evidence that intreferon acts intracellularly, which fits well with Isaacs' conception of the biochemical site of action of interferon(7). Additional data suggesting that S-VIF interferon acts *after* adsorption of polio-RNA and that treated cells do not preferentially inactivate RNA constitute further evidence favoring the intracellular site of action for interferon. More direct evidence with respect to adsorption of RNA by treated and untreated cells would be desirable. In our hands, however, measurement of adsorption of infectious RNA by the method of titration of residual activity has not proved as clear cut as in the work of Holland *et al.* Adsorption studies using isotopes may not be more informative because infectious RNA is difficult to label specifically(4).

In addition to inhibiting the infectivity of RNA, it is logically possible that interferon acts at a second site with respect to intact virus. This additional site of action could be where intact virus penetrates or where it uncouples into nucleic acid and protein components. This appears unlikely in view of the fact that degree of inhibition of RNA infectivity is as great if not greater than for intact viruses tested such as VSV. Ideally however, one should compare the inhibitory effect of interferon on one species of intact virus and its infectious RNA derivative.

There are advantages to the use of infectious RNA to study the kinetics of inhibition of interferon in a system in which the cells are resistant to the action of the intact virus progeny of the RNA. One advantage is that in such a system measurement of virus production is limited precisely to one cycle even when a small inoculum is applied. This would not be the case if instead intact virus such as VSV were used. Under these conditions, as virus production increases, the number of infectious cycles multiply and unsuspected factors such as secondary elaboration of viral inhibitors or even potentiators of infection may be added to the experimental conditions.

This study of the kinetics of inhibition of RNA infectivity brings out 2 interesting points. 1) Inhibition of virus production and inhibition of cell infection are of approximately the same magnitude. 2) Degree of inhibition of either virus production or infectious center formation does not increase linearly with concentration of the inhibitor. At high concentration of S-VIF, there is a rapid decline in rate of inhibition such that even with greater concentration of S-VIF, one may not expect complete protection of all cells in the presence of a large enough challenge inoculum. A similar rapid decline in degree of inhibition at higher concentrations of S-VIF using intact vesicular stomatitis virus was noted (Table I)(5). This suggests a limit to the amount of inhibitor that can be made available for cell protection. Such may be a characteristic of interferons in general or only of our particular preparation. Further kinetic data of this type including studies on adsorption of the inhibitor and secondary cell-inhibitor reactions would be desirable.

With respect to the first point, because of the fluctuations of inhibition measurements from one experiment to another, the data presented are inadequate to prove the suggested thesis that inhibition of cell infection alone can account for inhibition of virus production. Such fluctuations are partially due to the methodological difficulties inherent in the many steps necessary for titering infectious centers, and use of separate cell cultures for measuring cell infection and virus production in a particular experiment. Forthcoming improvements in titration methods may help overcome both of these difficulties. In addition, correlation with dose-response data using intact virus(1,5) will also be important.

*Summary.* An inhibitor with the properties of interferon obtained from harvests of Sindbis virus inhibits the infectivity of poliovirus RNA in chick embryo cell cultures. This inhibition is expressed by a reduction of cell infection as well as total poliovirus production. These results are compatible with the concept that interferon inhibits certain intracellular metabolic activities necessary for virus synthesis.

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Received May 18, 1961. P.S.E.B.M., 1961, v107.

# Immunological Relationship of Selected Feline Viruses by Complement Fixation. (26713)

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A number of viruses pathogenic for the domestic cat have been reported(1,2,3). In addition, a number of viruses of unknown clinical importance have been isolated from the nasopharyngeal region of domestic cats (4). The immunological relationship of a number of these agents has been established on the basis of serum neutralization tests in tissue culture(4,5). The purpose of this paper is to report the immunological relationship of certain viruses from both groups by means of the complement fixation test.

*Materials and methods.* The viruses selected for this study include feline viral rhinotracheitis (FVR)(2), kidney cell degenerating (KCD)(6), Bolin's panleukopenia (FPL)(7), California feline isolate (CFI)(3), and the feline viral isolates reported by Crandell as FRI 12, FRI 29, FRI 278(4).

Virus antigens for the complement fixation (CF) tests were prepared as follows: Viruses were grown in primary monolayer feline renal cell cultures prepared and maintained according to the method described by Madin(8). Viruses were harvested from 32-oz. culture bottles between 24-48 hours after inoculation. Harvesting consisted of storing the cells overnight at –20°C, thawing at room temperature, followed by centrifugation at 10,000 rpm for 10 minutes in a Servall SS-2 centrifuge to remove cellular debris. The supernatant was then centrifuged in a Spinco Model L at 30,000 rpm for one hour in a No. 30 rotor. The supernatant was discarded. The sediment was washed by resuspension in 0.02 M phosphate buffer pH 7.0 and recentrifuged in a like manner. The resultant pellet was resuspended to 1/10 the original volume, using 0.02 M phosphate buffer pH 7.0. This material is referred to in the text as 10X antigen and was used in the CF tests. Such material remains stable for periods up to 60 days at 4°C. Known positive sera were included in all tests to insure antigenic potency.

The antisera employed were obtained from 2 sources. The sera used to procure the data shown in Tables I and II were obtained from cats from which a specific virus had been isolated or from cats which had been infected with a single virus. The source is indicated in each table. Cats were inoculated under pentothal anesthesia by the intranasal route, using 0.25 to 0.50 ml of 30th tissue culture passage virus. Serum was obtained 30 days after inoculation.

The CF test employed in this study was a modified Kolmer technic using 0.1 ml amounts of antigen and antisera. Primary incubation was carried out at 4-8°C overnight. The usual controls were included in each test as well as a complement titration control series. Two units of complement (contained in 0.2 ml) as determined by titration in the presence of antigen were used. Block titrations were performed on all antigens, using at least 3 dilutions of antigen

TABLE I. Specificity of Immune Sera by Serum Neutralization Tests.

| | CFI antisera* | | FRI-278 antisera* | | FRI-12 antiserum† |
|---|---|---|---|---|---|
| Virus | 161 | 162 | 103 | 107 | 59–12 |
| FVR | <20‡ | <20 | <20 | <20 | <20 |
| CFI | *320* | *640* | <20 | <20 | <20 |
| FRI-278 | <20 | <20 | *160* | *320* | <20 |
| FRI- 29 | <20 | <20 | <20 | <20 | <20 |
| FRI- 12 | <20 | <20 | <20 | <20 | *640* |
| FPL | <20 | <20 | <20 | <20 | <20 |
| KCD | <20 | <20 | <20 | <20 | <20 |

* Obtained from cats inoculated with the virus indicated.

† Obtained from a cat from which FRI-12 virus was isolated.

‡ Reciprocal of highest serum dilution (final) that inhibited cytopathic change in 2 out of 3 tubes inoculated with serum virus mixtures.

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TABLE II. Immunological Relationship of Feline Viruses by the Complement Fixation Test.

| | CFI antisera* | | FRI-278 antisera* | | FRI-12 antiserum† |
|---|---|---|---|---|---|
| Virus | 161 | 162 | 103 | 107 | 59–12 |
| FVR | <4 | <4 | <4 | <4 | <4 |
| CFI | 256‡ | 256 | 256 | 64 | 128 |
| FRI-278 | 256 | 256 | 256 | 64 | 128 |
| FRI- 29 | 512 | 128 | 128 | 32 | 128 |
| FRI- 12 | 128 | 128 | 64 | 32 | 64 |
| FPL | 512 | 512 | 512 | 128 | 256 |
| KCD | <8 | <8 | <8 | <8 | <8 |

* Obtained from cats inoculated with the virus indicated.

† Obtained from a cat from which FRI-12 virus was isolated.

‡ Reciprocal of highest serum dilution showing positive fixation of complement.

and varying dilutions of known positive serum to determine the optimal antigen dilution. This optimal dilution of antigen was then used in all subsequent tests. Sera were inactivated at 56°C for 30 minutes. All titers are reported as the reciprocal of the highest dilution of serum showing positive fixation.

Neutralization tests were carried out by using primary feline renal cell tissue cultures. Between 100 and 200 TCID$_{50}$ of each virus, determined by replicate titration and calculated by the method of Reed and Muench(9), was employed. Equal volumes of serial serum dilutions were mixed with the test virus and allowed to stand for one hour at 24°C; 0.1 ml of each mixture was then inoculated into each of 3 tubes of tissue culture. Neutralization of the virus was indicated by the absence of a cytopathic effect (CPE). A virus control series was always included.

*Results.* The results of the serum neutralization test are shown in Table I and demonstrate the specificity of each of the antisera tested for its homologous virus. No cross neutralization was observed in any of the serum dilutions employed. These sera were then tested for CF antibody using 10X antigen described previously. The results are shown in Table II and indicate a serological cross reaction with 5 of the viruses tested. Two of the viruses failed to show any relationship.

*Discussion.* From the data shown in Tables I and II it would appear that each of the viruses is distinct when tested by serum neutralization and that 5 of the viruses appeared to be immunologically related according to the CF test. All of the viruses tested can arbitrarily be divided into 2 groups on the basis of inclusion body formation in feline renal cell cultures and time interval at which CPE occurs. Of these viruses, only FVR produces inclusion bodies in feline tissue culture and rate of CPE is slow when compared to the other feline isolates which do not produce inclusion bodies. The present study would tend to follow this line of separation immunologically since, with the exception of KCD, all of the other viruses inducing rapid CPE appear to be related, on the basis of the CF test. KCD virus was isolated by Fastier (6) in New Zealand and so might be expected to be serologically distinct from the other viruses.

Results similar to these have been obtained with 4 addiitonal viruses and their homologous antisera but are not included in the present report since these viruses have not yet been reported in the literature.

*Summary.* The relationship of 7 feline viruses was determined by means of the CF test. The results presented indicate that 5 of these viruses are antigenically related though immunologically distinct by the serum neutralization test in tissue culture.

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Received May 19, 1961. P.S.E.B.M., 1961, v107

# Studies on Binding of Glucose by Insulin.* (26714)

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These experiments were concerned with the possible binding of insulin and glucose. Levine and Goldstein(1), using rats, showed that insulin increased the distribution volume of sugars, the first 3 carbons of which were of the same configuration as glucose. This selectivity of insulin for sugars configurationally related to glucose suggested the possibility of a binding site complimentary to the first 3 carbons of glucose. It was felt that this hypothesis could be tested by dialyzing mixtures of glucose and insulin against saline at pH 7.4.

Insulin did not retard the free distribution of glucose across a semipermeable membrane. Therefore, it appears that insulin does not bind glucose sufficiently to affect its free distribution in solutions containing a physiological salt concentration and pH.

*Methods and materials. Crystalline beef insulin*[§] was weighed out and dissolved in 0.01 N NaOH, and to this was added an equal volume of 0.03 M Tris buffer, pH 8.9, (Tris-hydroxymethyl aminomethane). The pH of this solution was adjusted to 7.4-7.5 with 0.01 N HCl.

*Egg albumin* solutions (Armour's) used as the protein control were prepared in the same manner as the insulin solution. *Glucose* solutions were made up in distilled water. All solutions were prepared fresh for each experiment.

Dialysis for 48 hours gave the most consistent results. It was found that Visking 20/32 dialysis tubing passed insulin and that 12/32 tubing did not, as was previously observed by Craig and King(2).

Glucose was determined by the Glucostat method(3). All solutions were deproteinized with NaOH and $ZnSO_4$.

*Experimental procedure.* 1. 6 ml insulin (1 mg/ml) were placed in a dialysis bag with 1 ml of glucose (1.62 mg/ml) and dialyzed against 50 ml of normal saline at 2°C.

2. Number 1 was repeated using 1 ml of 2.4 mg/ml glucose instead of 1.62 mg/ml.

3. 6 ml of egg albumin (1 mg/ml) were placed in a dialysis bag with 1 ml of 1.62 mg/ml glucose and dialyzed against 50 ml of normal saline.

4. Number 3 was repeated using 1 ml of 2.4 mg/ml glucose instead of 1.62 mg/ml.

5. (Control) 3 ml of 0.01 N NaOH and 3 ml of Tris buffer were placed in a dialysis bag with 1 ml of 1.62 mg/ml glucose and dialyzed against 50 ml of normal saline.

All dialyses were done in 125 ml stoppered Erlenmeyer flasks in a 2°C water bath.

*Results.* Repeat experiments indicated that there was no appreciable difference between glucose concentrations inside and outside the dialysis bag in any of the glucose-insulin or glucose-egg albumin solutions tested. The initial glucose was accounted for in all experiments indicating no appreciable loss of glucose due to experimental manipulation or bacterial contamination.

There was no retardation of glucose distribution by insulin across a semipermeable membrane. Concentration of glucose within the bag was equal to concentration outside when dialyzed in presence of insulin. This was also noted when glucose was dialyzed in presence of egg albumin.

It appears that insulin does not retard glucose and probably does not combine with it.

*Discussion. Analysis of Table I*: If one mole of insulin were to bind one mole of glucose, there would have been 8 and 12.3 micromoles of glucose distributed on both sides of the semipermeable membrane in Flasks 1 and 2 respectively. Assuming this to be the case, there would have been 25.2

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* This work was supported by U.S.P.H.S. grant.

[†] U.S.P.H.S. Student Summer Research Fellowship.

[‡] Student Summer Research Fellowship sponsored by Southern Calif. Diabetes Assn.

[§] We wish to thank Dr. Otto K. Behrens, Eli Lilly, for the generous supply of crystalline beef insulin. (Lot #535664).

TABLE I.

| Flask No. | Location | Vol, ml | Glucostat determin'n, $\mu g$ | Predicted conc. on basis of free distribution | % of total glucose recovered |
|---|---|---|---|---|---|
| 1 | Inside bag | 7 | 28 | 27.5 | 98 |
| | Outside " | 50 | 27 | | |
| 2 | Inside " | 7 | 42 | 42 | 98 |
| | Outside " | 50 | 41 | | |
| 3 | Inside " | 7 | 31 | 27.5 | 100 |
| | Outside " | 50 | 28 | | |
| 4 | Inside " | 7 | 46.8 | 42 | 105 |
| | Outside " | 50 | 44 | | |
| 5 | Inside " | 7 | 28 | 27.5 | 101 |
| | Outside " | 50 | 29 | | |

$\mu g$ of glucose/ml in Flask 1 and 38.4 $\mu g$ of glucose/ml in Flask 2. This would have been the case if the glucose bound to insulin were *not* liberated when the solution was deproteinized. In this event only 89% of the glucose would have been recovered from Flask 1 and 92% from Flask 2 instead of the observed 98%.

If one mole of glucose were bound to insulin and liberated upon deproteinization, then there would have been an increase of 1 micromole of glucose within the bag in both Flasks 1 and 2. In this case there would have been 50.9 $\mu g$/ml instead of the observed 28 $\mu g$/ml in Flask 1 and 64.1 instead of 42 $\mu g$/ml in Flask 2.

All of the initial glucose was recovered, therefore, deproteinization liberated any glucose which may have been bound. Since there was no disparity between concentration of glucose inside and outside the bag, it is assumed that no glucose was bound. It would be inconsistent with the initial postulate to propose that less than one mole of glucose/mole of insulin were bound. In the light of these experiments it is assumed that, at physiologic ionic strength and pH, insulin does not bind with glucose.

*Summary.* Insulin does not retard distribution of glucose through a semipermeable membrane under the conditions of this experiment. Conditions in different media may show different results. It was decided not to investigate the initial premise further since the conditions of these experiments were similar to the pH and salt concentration encountered *in vivo*.

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Received May 22, 1961. P.S.E.B.M., 1961, v107.

## Development of a Strain of Psittacosis Virus Partially Resistant to 5-Fluorouracil in a Tissue Culture System.* (26715)

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The development of resistance to chemotherapeutic agents by psittacosis virus has been described(1-6). The present paper describes the development of resistance to 5-fluorouracil (FU) by strain TTF psittacosis virus in a tissue culture system.

*Materials and methods.* *"Wild-type" vi-*

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* Supported by funds from U.S.P.H.S. grant.

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TABLE I. Comparison of Sensitivity to 5-Fluorouracil (FU) between Wild-Type and FU-Resistant Strain of Psittacosis Virus.

| | | Virus units/ml produced in presence of FU at a concentration indicated below ($\mu$g/ml) | | | | |
|---|---|---|---|---|---|---|
| Virus phenotype | Exp. | 0 (control) | 1 | 2 | 3 | 4 |
| "Wild-type" | 1 | $1.1 \times 10^7$ | 4100 | $<10$ | — | — |
| | 2 | $3.3 \times 10^7$ | 1700 | $<10$ | — | — |
| | 3 | $1.1 \times 10^7$ | 2700 | $<10$ | — | — |
| FU-resistant strain | 1 ( 6th pass.) | $1.0 \times 10^7$ | $7.5 \times 10^5$ | $5.5 \times 10^5$ | — | 300 |
| | 2 ( 7th " ) | $2.3 \times 10^7$ | $1.2 \times 10^6$ | $4.5 \times 10^5$ | — | — |
| | 3 (10th " ) | $1.6 \times 10^7$ | $1.6 \times 10^6$ | $5.7 \times 10^5$ | $1.1 \times 10^4$ | 900 |

— = Not tested.

*rus stock.* The 34th passage of strain TTF psittacosis virus(7) in cultures of the McCoy cell line(8) represented the wild-type virus stock. The latent period of TTF virus in McCoy cell cultures was about 17 hours at 37°C and thereafter newly synthesized infective virus increased in number rapidly. Maximum yield of virus was reached by 48 to 72 hours after infection to a level between $5 \times 10^7$ and $10^8$ infective units per ml. The replication pattern and cytochemical changes associated with virus multiplication were reported elsewhere(9-11).

*Host cell.* A cellular derivative of human synovial tissue (strain McCoy) was propagated on coverslips in Leighton tubes, or in milk-dilution bottles. The nutrient medium consisted of Mixture 199 with 0.5% lactalbumin hydrolysate, 5% heat-inactivated calf-serum and 0.1 mg per ml streptomycin. The pH was adjusted to 7.2 by sterile sodium bicarbonate.

Infective virus units were assayed at 24 hours after inoculation by the "infected cell count method"(7,12).

*5-Fluorouracil*‡ FU was dissolved in distilled water (1 mg per ml), sterilized by Selas filtration and stored at –20°C. Graded amounts (25 $\mu$g to 5 $\mu$g/ml) were dissolved in nutrient medium and tested for effect on normal cells. Significant cytotoxicity was not observed at concentration of 25 $\mu$g/ml or less.

*Results. Sensitivity of TTF virus to FU.* Inhibitory action of FU on maturation of psittacosis virus was determined according to the procedures described in preceding experiments(13). Virus was inoculated, with a multiplicity of infection of 3, onto monolayer cell sheets on coverslips in Leighton tubes (approximately $5 \times 10^5$ cells per tube). Thus most of the cells were infected. After an interval of one hour, inoculated tubes were washed once and 1.5 ml of nutrient medium with graded dosages of FU was added to each. The infected cultures were then incubated at 37°C for 36 hours, during which time maturation of virus in FU-free control cultures was virtually complete, without extensive cell destruction. All cell cultures were then washed twice with fresh FU-free medium, and then with 2 ml of the same medium, each was subjected to repeated freezing. Infective virus units of such treated cultures were assayed by the infected cell count method.

The results of this experiment are shown in Table I and Fig. 1. FU inhibits maturation of the wild-type TTF virus completely at 2 $\mu$g per ml ($1.5 \times 10^{-5}$M). There is a linear dose-response relationship between concentration of analogue in the nutrient medium and log units of virus produced (Fig. 1).

*Isolation of FU-resistant viral strain.* Isolation of a virus strain which was resistant to FU was carried out by repeated selections and multiplications, as follows: Three ml of the wild-type virus ($3 \times 10^8$ infective units) was inoculated onto a bottle culture of McCoy cell. After absorption of one hour, 10 ml of the medium containing FU at 0.5 $\mu$g/ml was added. After incubation for 2 days at 37°C, the cell culture was washed with Mixture 199 and replaced with 10 ml of FU-free medium. The culture was twice

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‡ By courtesy of Dr. R. Duschinsky, Hoffmann-La Roche, Inc., Nutley, N. J.

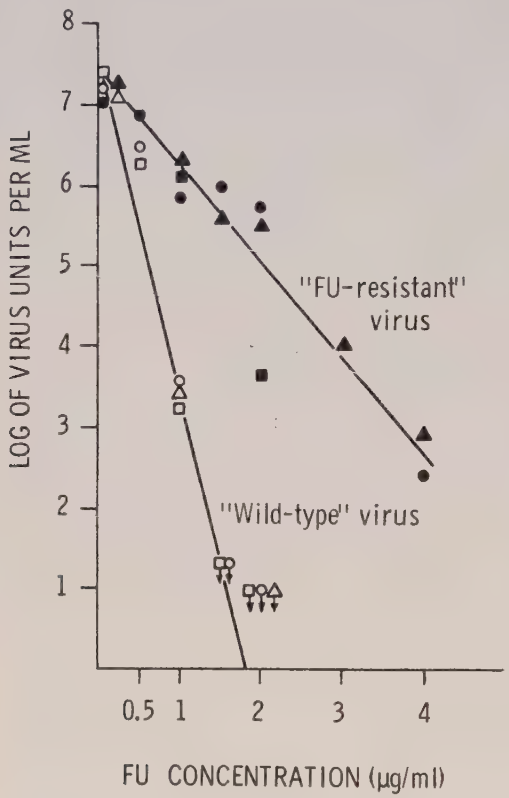

FIG. 1. FU-sensitivity of "wild-type" and FU-resistant psittacosis viruses. Virus levels produced in presence of FU are plotted as functions of analogue dosages. Symbols with downward arrows represent that the infective virus units were below a titer indicated by each point itself.

frozen and assayed. The cell culture thus prepared contained 5 × $10^5$ infective units per ml. Test of FU-sensitivity showed that the surviving virus was almost as sensitive to FU as the wild-type virus. This virus was inoculated into a bottle containing fresh cells (without FU) in which the propagating virus caused the cells to lyse after several days. The virus titer in the resulting virus stock (grown in FU-free nutrient medium) exceeded 2 × $10^7$ per ml. The latter virus stock was again inoculated into another bottle of McCoy cells and incubated with medium containing 0.5 μg FU/ml. After several cycles of selection-and-multiplication of virus as described above, virus acquired the capacity to mature in medium containing a borderline concentration of 2 μg FU/ml. Table I and Fig. 1 show the results of FU-sensitivity tests carried out with the 6th, 7th and the 10th passages. The FU-resistant strain produced more than $10^5$ units/ml of medium containing 2 μg FU/ml. Wild type virus was not produced in cultures which contained 2 μg FU/ml. It is apparent that the virus acquired some tolerance to FU during the serial passages in small amounts of the compound. In spite of further efforts, however, resistance of TTF virus to FU beyond the 2 μg/ml could not be demonstrated.

To determine the stability of the FU-resistance acquired by TTF virus, it was passaged through 5 series of cultures with FU-free medium, and again was propagated in medium with FU. A significant loss of FU-resistance was not observed. Thus, it may be concluded that the low level of FU-resistance of TTF virus was due to certain mutation(s) in virus genome rather than due to adaption.

*Discussion.* Cohen(13) and Heidelberger *et al.*(14,15) indicated that FU interfered with both RNA and DNA biosynthesis through its incorporation into RNA or by blocking the methylation reaction leading to formation of DNA-thymine, respectively. In addition, Skold(16) pointed out a direct inhibition of the pyrimidine nucleoside phosphorylase due to FU in the Ehrlich ascites tumor cells. Thus FU inhibits the synthesis of both RNA and DNA through at least 3 different processes.

Failure to isolate a virus strain completely resistant to FU may be attributed to the multiplicity of sensitive points by FU on biosynthesis of nucleic acid. The explanation that the acquired FU-resistance of psittacosis virus may be due to acquisition of FU-resistance by the host cell is readily discounted because virus was passaged always to fresh cells from cultures containing virus and killed cells.

The development of resistance to FU by psittacosis virus supports the claim that this microorganism possesses specific enzyme systems for synthesizing viral materials, especially for biosynthesis of viral nucleic acid, which are, at least in part, different from those of host cell itself.

*Summary.* A strain of psittacosis virus (TTF) developed resistance to 5-flourouracil (FU) after serial passages of the original FU-sensitive virus in tissue culture cells with small concentration of FU in the medium. The FU-resistant strain of TTF virus multiplied and produced more than $10^5$ infective units per ml in presence of 2 μg FU/ml, at which level FU suppressed the original wild-type TTF virus completely. Reversion of character from FU-resistance to FU-sensitivity following serial passages in FU-free medium was not observed. This suggests that the acquisition of FU-resistance is due to certain gene mutation(s), rather than to enzymatic adaptation.

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Received May 22, 1961. P.S.E.B.M., 1961, v107.

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## Comparison of Assays for Mengovirus and Reovirus-3.* (26716)

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Reoviruses were recently recognized as a group distinct from enteroviruses and other well known groups of animal viruses(1). In view of the susceptibility of weanling mice to these viruses(1), it appeared reasonable to determine whether reoviruses would grow and produce cytopathic effects in L-cells, a well defined cell line originally derived from mouse connective tissue. This paper describes the conditions for plaque assay of reovirus-3 and compares these conditions with those for the plaque assay of Mengovirus in L-cells, a virus which has been studied in this laboratory in recent years.

*Materials and methods.* L-cell, strain 929, originally obtained from Microbiological Associates, Inc., was cured of PPLO infection with kanamycin(2) and maintained free of PPLO by continued use of kanamycin in the cell stock cultures. The medium for stock cultures consisted of Eagle's minimum essential medium (MEM)(3) supplemented with 5% horse serum. Serum from a single selected horse was used, since sera from some horses are cytotoxic. The following antibiotics were used in the stock cultures: penicillin (500 units/ml), streptomycin (0.5 mg/ml), mycostatin (25 units/ml), and kanamycin (0.5 mg/ml). When stock cells were plated for any biological studies or plaque assays, the same nutrient medium was used, but only streptomycin and penicillin were present.

Mengovirus was obtained from Dr. M. Theiler as a frozen mouse brain suspension, stored since 1955. This virus was originally isolated by G. W. A. Dick in Uganda(4). Reovirus-3, strain Dearing, was obtained from Dr. H. J. Eggers as the 10th monkey kidney cell passage. This virus was isolated by M. Ramos-Alvarez in Cincinnati(5).

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* Aided by a grant from the National Foundation.

Plaque assays were performed as follows: Virus was diluted in MEM. Monolayers of L-cells grown in 60 mm plastic Petri plates (Falcon Plastic Co.) were washed once with MEM and 0.3 ml of an appropriate virus dilution was added. The virus was allowed to adsorb for an adequate time (see results) at 37°C in 5% $CO_2$. The inoculum was redistributed over the monolayer every 10 minutes. The agar overlay consisted of an enriched modification of MEM containing 5% horse serum(6). Cells were stained with neutral red agar after several days of incubation.

*Results.* Reovirus-3 was passaged in monolayers of L-cells, the very first passage resulting in a rapid cytopathic effect. Two more passages were made and then a plaque assay was attempted on monolayers of L-cells. This proved successful and therefore the conditions for optimal plaque formation were examined and compared with the conditions for Mengovirus plaque formation. Table I indicates an increase in plaque number from day 8, when some plaques are just visible, until day 14. No further increase was noted up to day 20. In contrast Mengo plaques are visible 2 or 3 days after inoculation and there is no increase in number of plaques after 4 days. The plaque size of uncloned reovirus stock ranges from 0.1-1.0 mm in diameter on day 9, whereas the plaque size of uncloned Mengovirus ranges from 1.0 to 8.0 mm in diameter on day 8. Large and small plaque variants have been isolated from Mengovirus and a cloned line of moderately uniform plaque size has been isolated from reovirus-3.

TABLE I. Variation of Reovirus Plaque Titer with Time of Incubation. Cells were exposed to 30-100 plaque forming units for 1 hr and overlaid with nutrient agar. After 7 days of incubation the cells were stained with neutral red agar and plaques were counted on subsequent days.

| Day after inoc. | Avg PFU/ml |
|---|---|
| 8 | $1.30 \times 10^8$ |
| 9 | $1.33 \times 10^8$ |
| 10 | $1.80 \times 10^8$ |
| 14 | $2.33 \times 10^8$ |

TABLE II. Comparison of Plaque Assay and Limiting Dilution Assay for Mengovirus and Reovirus-3.

| | Plaque assay* (PFU/ml) | Limiting dilution assay† (infectious units/ml) |
|---|---|---|
| Mengovirus | $1.02 \times 10^8$ | $1.96 \times 10^8$ |
| Reovirus-3 | $1.89 \times 10^8$ | $2.77 \times 10^8$ |

* Plaque assays on L-cell monolayers. Mengovirus plaques were read on 4th day and reovirus plaques on 9th day.

† Mengovirus was assayed in 6-8 wk old mice by standard intracerebral inoculation. Reovirus was assayed in primary cultures of rhesus monkey kidney. $LD_{50}$ or $TCID_{50}$ was determined by probit analysis and infectious units calculated by multiplication of the $LD_{50}$ or $TCID_{50}$ by 0.69.

A second variation in plaque assays is the length of time allowed for adsorption of virus. This was studied by exposing monolayers to 50-100 PFU of virus in 0.3 ml MEM at 37°C in 5% $CO_2$ for varying periods of time, after which the inoculum was removed and the cells washed twice with MEM before addition of nutrient agar. The results shown in Fig. 1 indicate that maximum amount of

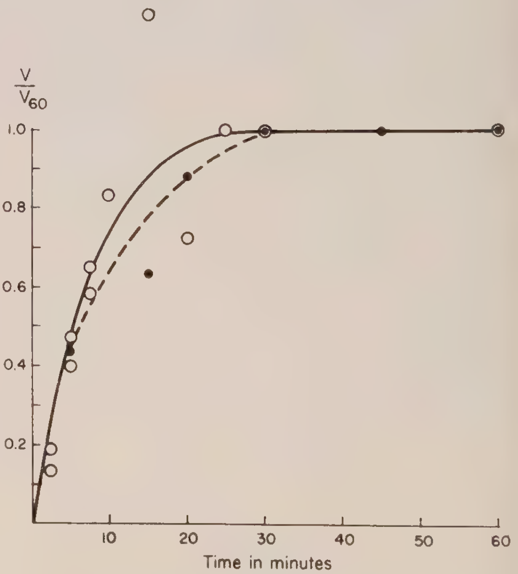

FIG. 1. Adsorption of Mengovirus (○) and reovirus-3 (●) to monolayers of L-cells at 37°C. The fraction of maximum amount of PFU adsorbed is plotted *vs* time. This fraction is calculated from ratio of PFU at time t to that at 60 min. ($V/V_{60}$).

each virus adsorbed in 30 minutes. This time has therefore been adopted in assays of both viruses.

The plaque assay of Mengovirus was compared with the assay in mice and the plaque assay of reovirus with the 50% infective endpoint titration in monkey kidney cultures. The plaque-forming units of both viruses are very similar in number to the infectious units in the $ID_{50}$ assays. Reovirus plaques were read on the 9th day; corrected to the 14th day gives a titer of $3.3 \times 10^8$ PFU/ml. Thus it appears that plaque assays of both Mengovirus and reovirus-3 are as sensitive as $ID_{50}$ assays and that repeated passage in L-cells does not result in loss of virulence for mice in the case of Mengovirus or for monkey kidney cells in the case of reovirus.

*Summary.* Reovirus-3 was passaged in L-cells where it grew and produced a cytopathic effect. A plaque assay in L-cells is described for this virus and compared with the plaque assay for Mengovirus.

Miss Joan Callender provided excellent technical assistance.

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Received May 25, 1961. P.S.E.B.M., 1961, v107.

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# Relationships of Testicular Cholesterol to Hormonal Activity and Behavior in the Starling.* (26717)

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Androgenic hormones are known to influence very dramatically behavior associated with courtship and breeding in many birds (1). In the British starling Bullough showed that these behaviors were greatly intensified during the period of most rapid growth of the testes(2). Witschi and Miller found that the color of the bill in both sexes of the starling is controlled by testosterone (3). As the gonads increase in mass the bill changes from black to yellow, and as the testes decrease in weight the bill returns to the black condition.

Cholesterol has been shown to be an important precursor in the biogenesis of the steroids of adrenals and testes(4,5,6). Marshall and others have observed histochemically changes which occur in the cholesterol-positive lipoids of avian testes during the annual cycylic activity(7). The present study conducted on starlings correlates the cyclic changes in mass of the testes with quantitative changes in concentration of cholesterol present in the organ. An hypothesis is advanced concerning the relationship of cholesterol levels to steroid hormone production. Correlations between behavioral activity of the starlings and general histological condition of the testes are also reported.

*Material and methods.* Starlings from a known stage in their behavioral cycle were killed with a shotgun. They were autopsied immediately and the testes were removed, blotted and weighed on a Roller Smith balance, and frozen in glass vials imbedded in dry ice. Cholesterol content of the testes was determined by the method of Knobil, *et al.*(8) and reported as total cholesterol since the free and esterified forms were not sepa-

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* The data presented here were submitted to the School of Hygiene and Public Health, Johns Hopkins University, as a portion of a dissertation for the degree of Doctor of Science. This work was supported by N.I.H. Division of Research Grants, U.S.P.H.S.

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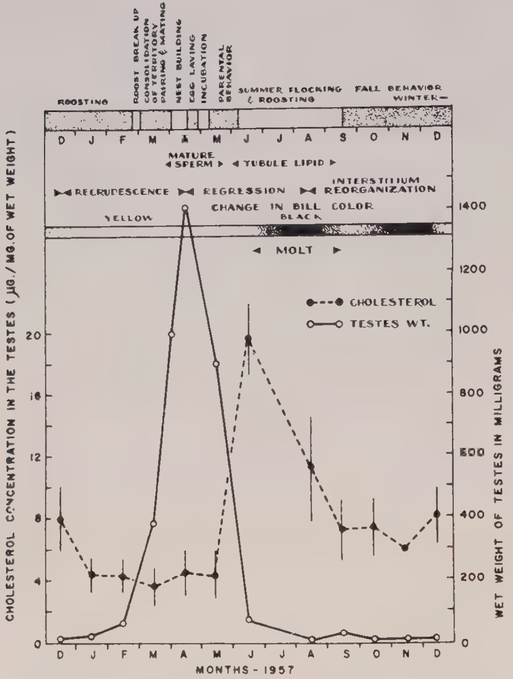

FIG. 1. Yearly cycles of behavior, weight and condition of testes, color of bill and concentration of cholesterol in testes of starlings.

rated. Tissue for histological study was preserved in Bouin's fluid.

*Results.* The results are summarized in Fig. 1 in which concentration of cholesterol in $\mu$g per mg of testes is plotted as the mean monthly value (dashed line) plus or minus 2 standard deviations. The variance was omitted for the November data since determinations from only 3 individuals were made during that month. A total of 110 starlings was used in the entire study and, with the exception of November, mean value for each month was based on analysis of between 8 and 10 individuals. The decrease observed in concentration of cholesterol between December and January is statistically significant ($P<.01$), as is the increase in concentration between May and June ($P<.01$). The marked decline between July and September is also statistically significant ($P<.01$). The solid line represents mean monthly weights of the testes used for the determinations. Across the top of the figure the time of molt, bill color change, and histological condition, as well as the various behavioral periods are indicated.

*Discussion.* While relatively little histological information for these particular starlings is available, a generalized pattern can be adapted from work on the starling testes by Bissonnette(9,10) and on other avian species by Marshall(11). It is apparent that certain correlations exist between the histological condition of the testes, cholesterol concentration in the testes, and annual cycle of behavioral and physiological changes in the starling.

The first important correlation to be considered is the decrease in cholesterol concentration which accompanies the very earliest onset of testicular recrudescence (December-January). The rapid decline in concentration is followed by a period of 4 months during which cholesterol concentration is constantly maintained at its lowest level. During this time the level of behavioral and physiological activities reaches a maximum and culminates in breeding. Presumably the hormonal secretions of the testes are primarily responsible for eliciting these responses. Rosenfeld(12) has shown that in the mammalian adrenal cholesterol concentration drops markedly when output of corticoids rises. If such is true of the testes of the starling this should be the time of maximum androgen production and/or utilization. It is suggested that the observed decline in concentration of cholesterol could be due to an increased use of this precursor molecule for the biogenesis of more testicular hormone.

A second correlation is observed during the latter phase of testicular regression in July and August when cholesterol concentration increases markedly at the time when cholesterol appears in the tubules. This might suggest non-use, altered or greatly reduced use of the cholesterol molecule in biosynthetic pathways. Lofts and Marshall (13) have shown in the spermatogentically active or recrudescing testes of the pigeon that the androgenic steroids androsterone and androstenedione are present in the interstitial tissue. In the regressing testes these androgenic hormones disappear and the lipid is found in the degenerating seminiferous tubules. From the intra-tubular lipid Lofts and Marshall have isolated progesterone and other progestins as well. It is also known that progesterone induces molt in some birds. Direct proof that in the starling the high concentration of cholesterol found in the regressing testes plays a role in production of progesterone or other steroid hormones is lacking. However, it is interesting to note the correlation that exists between onset of the molt and occurrence of the highest concentrations of cholesterol.

A third correlation is observed when the accumulation of cholesterol-positive material in the tubules slowly disappears and concentration decreases until it reaches a plateau during the start of fall territorial behavior. The amount of androgenic hormone present and/or utilized during the fall and winter months appears to be less than that available during the period of intensified spring activity, but more than that operative during the low ebb of behavior observed in July and August.

An hypothesis, based on the observed concentrations of cholesterol present throughout the annual testes cycle of the starling, can be illustrated by 3 phases: (a) The lowest cholesterol concentration, greatest hormone production and most active period of behavior are observed during courtship and breeding. (b) The highest cholesterol concentration, lowest hormone production, and period of least behavioral activity found at time of summer flocking. (c) Intermediate concentrations, moderate hormone production, and moderate behavioral activity are shown to accompany fall and winter territorialism.

*Summary.* Levels of cholesterol were determined in testes of starlings collected from all portions of the gonadal and behavioral cycle. The lowest concentration of cholesterol was observed during recrudescence of the testes and at the time of most active behavior. The highest concentration was observed during regression of the testes and at the period of lowest behavioral activity. Intermediate concentrations of cholesterol accompanied the moderate behavioral activity observed in fall and winter. An hypothesis relating cholesterol concentration, hormone production and behavioral activity is advanced.

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Received April 28, 1961. P.S.E.B.M., 1961, v107.

# Encephalomyelitis and Pneumonitis in Hamsters Infected with Newcastle Disease Virus by Different Routes.* (26718)

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Intracerebral injection of Newcastle disease virus (NDV) has been shown to cause encephalomyelitis in hamsters(1) and monkeys(2). Similarly, mice(3,4) and cats(5, 6) develop encephalomyelitis, pneumonitis or both following intracerebral or intranasal administration of this virus. Among mammals, there have been no reports of successful infection with this virus administered by routes other than these. The purpose of this communication is to describe the pattern of disease and pathological findings of encephalomyelitis and pneumonitis in hamsters caused by NDV injected by peripheral routes.

*Materials and methods.* The virulent chicken-adapted CG strain of NDV was obtained from F. B. Bang. The pool of infected allantoic fluid harvested 48 hours after inoculation of 11-day-old embryonated eggs and used in these experiments titered $10^{8.2}$ plaque forming units (PFU) per ml in chick embryo fibroblasts.

The virus, diluted in phosphate buffered saline, was administered by either the intranasal (0.05 ml); intracerebral (0.05 ml), intramuscular (0.15 ml), subcutaneous (0.10 ml) or intraperitoneal (0.5 ml) routes to 21-day-old hamsters obtained from Lakeview Farm, Newfield, N. J. Virus was administered intranasally by dropping diluted inocula into the external nares of anesthetized animals and hyperextending the head to allow adequate contact of the nasopharyngeal mucosa with infective material. Intracerebral injections were made through the mid point of the skull. Subcutaneous, intraperitoneal, and intramuscular inoculations were made into the interscapular region, through the lower right abdominal wall and into the right thigh respectively. In each experiment, sterile diluent fluid was administered to a control group of hamsters by the same route as the virus.

The hamsters were placed in individual cages and examined daily during a period of 3 weeks for signs of illness or death. Partial autopsies were performed on nearly all animals and complete autopsies were made on the 179 animals examined histologically. Tissues were fixed in 10% formol-saline. Brains were embedded in celloidin and sectioned coronally or sagittally at 20 μ and stained with cresyl violet or hematoxylin and eosin. Other organs were embedded in paraffin, sectioned at 8-10 μ, and stained with hematoxylin and eosin.

In some experiments, brains and lungs were removed aseptically, ground in Ten Broeck grinders, and diluted to 10% (W/V) with buffered saline (0.01 M phosphate, pH 7.3). Virus isolations from tissue homogenates were made by subinoculations of 180 mm × 16 mm tube cultures of chick embryo fibroblast or 10-day-old embryonated eggs. The chick fibroblast cultures were inoculated 2 days after seeding, and maintained with mixture 199 and 10% calf serum for a week. Tissue culture fluids collected from cultures showing cytopathic effect were also tested for their capacity to agglutinate chick red blood cells. Ten-day-old embryonated eggs were injected into the chorioallantoic sac and observed daily for 5 days. Allantoic fluid collected from dead embryos was also tested for presence of hemagglutinins.

*Results.* The results of several titrations of NDV shown in Table I are expressed in terms of average number of PFU required to produce death in 50% of hamsters receiving virus by each route. As noted, hamsters were extremely susceptible when injected intracerebrally and least susceptible when in-

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* Aided by grants from USPHS and the Whitehall Foundation.

† Kenny Foundation Scholar.

TABLE I. Number of Plaque Forming Units Required to Produce Encephalomyelitis by Different Routes of Inoculation.

| Route of inoc. | No. of animals | PFU/LD$_{50}$ |
|---|---|---|
| Intracer. | 199 | .30 |
| Intranasal | 182 | 5.70 |
| Intraper. | 358 | $3.9 \times 10^3$ |
| Intramusc. | 208 | $4.4 \times 10^6$ |
| Subcut. | 212 | Indeterminate |

jected by the subcutaneous and intramuscular routes.

The mean time of death of animals which died of NDV infection in each experiment is shown in Fig. 1. For each route of inoculation there was a correlation between amount of virus injected and mean time of death. In view of the observation that few animals died after the 12th day following administration of virus, it may be concluded that if an animal survived this period the probability of survival beyond the 3 weeks of observation was excellent.

*Effects of physiologic stress.* The effect of physiological stress on susceptibility of hamsters to NDV was tested in 2 ways. In one series of experiments, NDV was titered in hamsters kept in controlled temperature rooms maintained at 13°-15°, 21°-23°, or 28°-30°C. In another series of experiments, NDV was titered in hamsters made to swim for 1 hour, at different intervals after infection, in individual beakers containing water at 36°-38°C. In none of these experiments was there any difference observed in the signs of disease, pathology of lesions, or in amount of virus required to produce illness.

*Pattern of disease.* Hamsters infected with lethal doses of NDV, regardless of route, developed signs of encephalomyelitis, pneumonia, or frequently both. The onset of illness was characterized by hunching of the trunk, ruffled fur and inactivity. Some animals when disturbed exhibited hyperexcitability. Left alone they were quiet. These initial signs were followed by development of segmental or generalized jerking movements of the head and neck, jaw, trunk, or fore and hind quarters (myoclonus). The rate of these predominantly flexor spasms varied from 40-160 per minute. Paralysis of one or more limbs was often observed; terminally, the animals were usually prostrate. With progression of the illness, respirations became labored and in those animals with generalized myoclonus the respiratory rate became synchronous with the myoclonic spasms. Death usually occurred one to 3 days after onset of the first signs of illness. In some instances, animals were found dead in their cages without previous signs of illness.

TABLE II. Results of Histological Studies of Hamsters Administered NDV.

| Route of infection | Animals examined | Encephalitis | Pneumonitis |
|---|---|---|---|
| Intracer. | 50 | 32 | 44 |
| Intranasal | 53 | 25 | 43 |
| Intraper. | 46 | 22 | 42 |
| Subcut. | 30 | 11 | 25 |

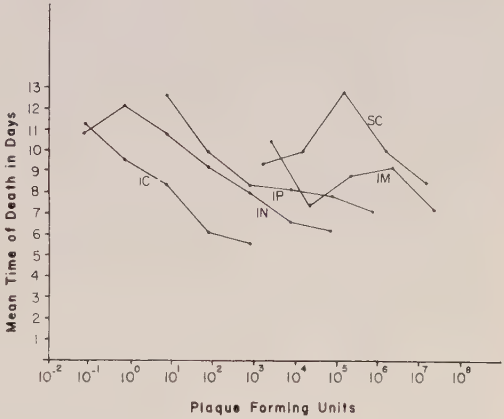

FIG. 1. Relationship between virus dose and mean time of death.

The predominant manifestations of encephalomyelitis in these animals were myoclonus and paralysis, both of which were observed in approximately 70% of animals that died. In general, there was a correlation between number of animals which developed myoclonus and paralysis and dose of virus administered.

*Histopathology.* Semi-serial sections of brains and single sections of lungs of hamsters dying after infection with NDV were examined and compared with those from control animals. The number of brains and lungs showing histologic evidence of encephalomyelitis or pneumonitis, respectively, is shown in Table II.

Perivascular cuffing was the most prominent histologic lesion of the brain irrespective of route of infection. This finding was most evident in the midline nuclei of thalamus, hypothalamus, tegmentum of the mid brain and brain stem, and basal cerebellar nuclei. In addition, brains of animals with more advanced perivascular cuffing showed considerable vascular dilatation. This was sometimes associated with red cell diapedesis into the neural tissue. Glial nodules and focal inflammatory changes were found in these same regions in brains with more severe encephalomyelitis. Specific neuronal lesions were rare. Except for an occasional animal with focal meningitis and contiguous perivascular spread into the brain, the cerebral and cerebellar cortices were essentially normal.

In animals which received NDV by the intranasal route, cuffing of blood vessels, glial nodules and focal inflammatory changes were found in the olfactory bulb and tract, the preoptic areas and fornices. The amygdaloid nuclei, paraolfactory and septal areas, and the cornu ammonis showed similar lesions.

In the lung sections of those animals with pneumonitis, there was evidence of severe passive congestion. The alveoli were commonly filled with edema fluid or edema fluid and blood. Interstitial infiltrations of mononuclear cells and erythrocytes and patchy atelectasis were observed. Hemorrhagic consolidation of entire lobes was a frequent finding regardless of route of virus inoculation. Epithelial degeneration was most prominent in the hilar bronchi, but in more advanced stages of pneumonitis, this process extended into the smaller bronchi.

*Virus recovery.* Attempts were made to recover virus from brains and lungs of sick animals. Ten-fold dilutions of brain or lung homogenates were inoculated into cultures of chick embryo fibroblasts and embryonated eggs. Virus was recovered from brains but not from lungs of 12 animals, injected 6 each by the intracerebral and intraperitoneal routes respectively. The average titer of virus recovered from brains in embryonated eggs was less than $10^3$ 50% egg infectious doses.

In other experiments no virus was recovered in embryonated eggs from the blood of hamsters inoculated with varying doses of NDV by the intraperitoneal route and collected immediately after inoculation and daily for 4 days.

*Uninfected Animals.* Of 214 animals which received sterile diluent inoculated by different routes, 27 died during the 3 week period of observation. Of these animals, 6 died from diarrheal disease and rectal prolapse. Nine showed histological evidence of pneumonitis. In the remaining 11, cause of death was not established. None of the control animals given sterile diluents developed encephalomyelitis.

*Discussion.* The significant finding of this study was the production of encephalomyelitis in hamsters following inoculation of the chicken-adapted CG strain of NDV by peripheral routes. The manifestations and pathology of the disease were similar in all respects to that produced by intracerebral injection of this virus. Encephalomyelitis, pneumonitis or both developed 5 to 13 days after inoculation. Length of the incubation period was related to dose and route of virus administration. The results of the present observations indicate that the hamster is more susceptible to disease produced by this virus than any mammal yet studied.

Demonstration of viremia as an essential precursor to many naturally acquired and experimentally induced viral encephalomyelitides raises important questions about exact sites and conditions favoring virus penetration into the CNS. Judging from the prominent perivascular cuffing and vascular congestion in the CNS of hamsters with NDV encephalomyelitis, it is suggested that the primary action of NDV in the hamster is on the blood vessels rather than nerve cells.

The experiments in which inoculated hamsters were maintained in controlled temperature rooms or made to swim for varying lengths of time were designed to determine whether physiological stress could alter the amount of virus required to kill 50% of animals, or increase the probability of infectious particles making contact with susceptible cells in the CNS. The failure of these experiments would seem to indicate that either the physiological stresses were inadequate, or at the time at which they were applied the virus was absorbed by other tissues and was no longer available to the CNS. It is of interest, in this connection, that no virus was recovered in the prodromal period from blood or lungs; very little virus was recovered from brains. From the finding that very little virus is required to initiate infection in animals by intracerebral inoculation, it may be concluded that there is an abundance of susceptible cells in the CNS; but their capability to produce virus is of a relatively low order as compared to chick cells in ovo or tissue culture. The amounts of NDV recovered from brains of infected hamsters were of the same order of magnitude as those amounts recovered from brains of other mammals(4).

From the observation that several hamsters inoculated with sterile diluents developed pneumonitis, it is possible, although evidence is lacking, that the inoculum may have provoked the multiplication of a latent infectious agent other than NDV. The pneumonitis in the inoculated animals, therefore, may be due to either: a) NDV infection despite poor recovery of virus from the lung tissues. b) accumulation of cytotoxic products of cells infected with NDV as suggested by others(7), or c) multiplication in the lung of a latent agent which interfered with multiplication of NDV.

*Summary.* Hamsters injected intracerebrally or by peripheral routes with NDV developed encephalomyelitis and pneumonitis. The amount of virus required to produce infection varied for each route, being least by the intracerebral route and highest by the subcutaneous route. Virus was recovered from the brain but not from the lungs or blood in the prodromal stages. The manifestations and pathology of the resultant encephalomyelitis were similar irrespective of route of inoculation.

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Received May 11, 1961. P.S.E.B.M., 1961, v107.

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## Postsystolic Myocardial Augmentation: 1. Effect Upon an Induced Shock State.* (26719)

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Different types of mechanical assistance have been considered in management of central circulatory insufficiency. Four major maneuvers have been considered: (1) total or partial cardiopulmonary bypass; (2) postsystolic myocardial augmentation; (3) veno-arterial shunt; and (4) selective right or left ventricular bypass. In postsystolic myocardial augmentation the augmenting ventricle (Fig. 1) is a blind-ended diverticulum which aspirates blood during the true ventricular systole and returns the blood to the arterial tree during true ventricular diastole, when the aortic valve is closed. Thus, the ventricle contracts against reduced resistance. Such counter-cycling can reduce and

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* This work was made possible by a grant-in-aid from Mr. Thomas A. Larkin of New York City and Research Contract from Office of Surgeon General, U. S. Army.

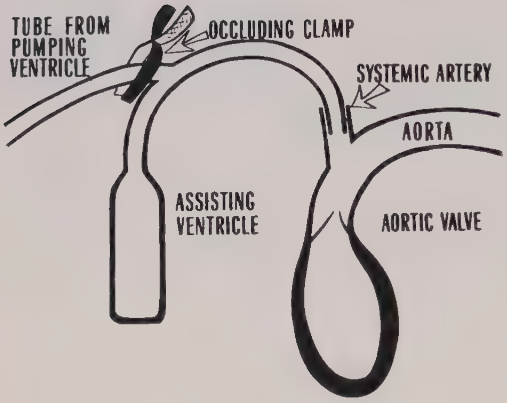

FIG. 1.

perhaps eliminate the intraventricular pressure curve. The real problem lies in phasing and synchronization of the augmenting ventricle. We have developed such equipment and an electronic circuit which, utilizing the QRS complex of the electrocardiogram, enables the phasing of the augmenting systole to be accurately placed.

*Methods.* In the initial phase of this investigation it was necessary to ascertain the most suitable arteries for cannulation according to the effect which such cannulation would produce upon the central pulse. Fifty-two dogs, weighing from 15 to 30 kg, anesthetized with 30 mg per kilo of pentobarbital, and heparinized with 1 mg per kilo, were used. Experiments were so designed that cardiac output and blood volume changes could be recorded directly in conjunction with phasic changes in blood pressure.

The arteries used for connection with the augmenting ventricle were cannulated with graded, tapered plastic catheters. The connection between the animal and the augmenting ventricle was achieved with rigid Tygon tubing, and the system was primed with 50 to 100 ml Ringer's solution. To obtain information as to the pulse form in different phases of postsystolic augmentation, since many such pulse tracings may be complex to interpret, the largest vessel available should be used. The abdominal aorta was cannulated with a large, stiff cannula just rostral to its bifurcation.

The effect of cannulating the aorta was closely approximated by cannulating both femoral arteries simultaneously. In the human, the femoral vessels are large enough to give adequate effect on the pulse when cannulated through the profunda femoris vessels of one or both sides, and there is no need to seek other vessels. After the preliminary attempts upon dogs, the abdominal aorta was selected, to establish the necessary parameters. The aortic cannula was connected to the augmenting ventricle by a short Tygon tube one-half inch in diameter, and the ventricle was seated in its actuator. The actuator was electrically triggered in the usual fashion; the subsequent aortic pulse recorded and studied through a recording cannula attached to a Statham P23B strain gauge, recorded upon the oscilloscope, then photographed with a modified Polaroid Land camera so that immediate permanent records were obtained. This pulse tracing was recorded simultaneously with the ECG tracing which also recorded the phase of the triggering of the assisting ventricle through an electronic circuit designed to show this point as an upright line following the QRS wave and named by us the "A" wave (Fig. 2).

Intravascular pressures measured with Statham P23B gauges were recorded through plastic catheters introduced into the left ventricle through puncture of the wall of the left atrium in the open-chest dog, or into the aortic arch through a carotid artery. Vena caval pressures were measured utilizing a catheter passed through the external jugular vein. Esophageal temperature was maintained at a normal level. In those experiments in which left ventricular pressure was determined or in which the subclavian artery was used for connection with the augmenting ventricle, the open-chest preparations were used, and ventilation was accomplished using a modified Vivian Thomas positive pressure respirator.

The augmenting ventricle was that of the Davol pump, which is a ventricle-type pump with a passive valve. This pump incorporates means for synchronizing the action of the pumps with any EKG signal or pacemaker, a feature of particular interest for augmented circulation. Mere synchronization of the pump with the EKG signal would not make possible the desired end results since the R wave merely indicates onset of systole. Because the mechanical action of the heart and the subsequent hydraulic end results in the aorta are not simultaneous with onset of systole, it was necessary to devise means not only for synchronizing the pump with the EKG but also a circuit so as to provide the proper phasing of the action of the pump with the hydraulic events of the aorta. By controlling the synchronization and, at the some time, controlling air pressure in the exhaust to determine the rate at which systole and diastole are made, it is possible to provide a synchronized pulse pattern of any desired form. Although at this time there is apparently no evidence which demonstrates the superiority of pulsatile flow over non-pulsatile flow or *vice versa,* there can be no argument that if the systolic pulse pressure is reduced, the work load of the heart is also reduced.

Twenty control dogs were prepared as follows. An infusion of norephinephrine was administered to dogs from which one-third of total calculated blood volume was removed before beginning infusion. Norepinephrine was administered in such dosage and at such speed over a 40 minute period that the systemic arterial blood pressure was elevated to a level from 220 to 240 mm/Hg during the period. All of these control animals died within 2 hours. Examination disclosed a diffuse hemorrhagic myocarditis. Details of this preparation will be reported elsewhere. An experimental series was designed in which the animals were placed upon postsystolic myocardial augmentation following production of the lethal shock state.

*Results.* The results obtained in a representative group of dogs subjected to postsystolic myocardial augmentation are shown in Table I.

In the 5 examples cited there is no essential difference in cardiac output of an individual animal before or after being placed upon postsystolic myocardial augmentation (last column, Table I). The decrease in left intraventricular pressure in all cases in which the animal was undergoing augmented circulation is significant. The elevation of arterial blood pressure measured in the aorta is appreciably higher during augmentation than during the control period. The column headed VC, or ventricular component, demonstrates that portion of the pressure curve which is contributed by true ventricular contraction. It is apparent that in the physical system in which cardiac output remains constant but in which intraventricular pressure is reduced, the work of the heart must also be reduced.

TABLE I.

| | $O_2$, cc/min. | A/V $O_2$ diff., cc/100 | LVP mm Hg | AP mm Hg | VC mm Hg | CO/ min. |
|---|---|---|---|---|---|---|
| A | 110 | 3.5 | 164/0 | 158/120 | | 3.124 |
| | 112 (PSA) | 3.5 | 50/6 | 250/120 | 38 | 3.200 |
| B | 146 | 8.8 | 160/0 | 164/132 | | 1.859 |
| | 144 (PSA) | 7.9 | 90/2 | 240/128 | 36 | 1.714 |
| C | 84 | 3.1 | 148/2 | 144/119 | | 3.709 |
| | 80 (PSA) | 2.8 | 100/0 | 214/130 | 21 | 2.857 |
| D | 154 | 4.2 | 160/0 | 158/130 | | 3.666 |
| | 142 (PSA) | 4.0 | 95/5 | 250/130 | 34 | 3.550 |
| E | 106 | 5.2 | 168/0 | 164/129 | | 2.038 |
| | 104 (PSA) | 5.1 | 83/2 | 220/110 | 38 | 2.132 |

$O_2$, cc/min. = oxygen consumption, ml $O_2$ STPD /min.

A/V $O_2$ = difference in cc/min. ml $O_2$ STPD/100 ml blood.

LVP = left intraventricular pressure.

AP = systemic arterial pressure.

VC = ventricular component—that portion of resultant pressure curve contributed by contraction of true ventricle.

CO/min. = cardiac output (calculated by direct Fick principle), l/min.

Table II shows representative results in 5 animals which were subjected to hemorrhage and administration of an infusion of norepinephrine as described under *Methods.* The marked hypotension following this

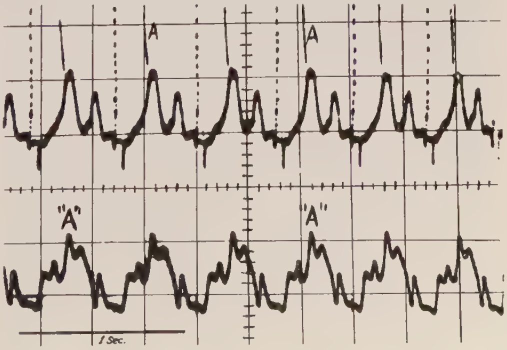

FIG. 2.

TABLE II.

| Initial AP, mm/Hg | AP ff cessation, norepinephrine and hemorrhage | Pressure after 2 hr PSA | Survival time, hr |
|---|---|---|---|
| 155/130 | 40/30 | 145/ 60 | 3 |
| 188/130 | 45/40 | 140/ 60 | 4 |
| 178/149 | 55/45 | 134/ 64 | 5 |
| 160/138 | 50/45 | 190/ 88 | 48 |
| 166/140 | 43/40 | 163/100 | 5 |

AP = systemic arterial pressure.
PSA = postsystolic myocardial augmentation.

maneuver is illustrated; elevation of blood pressure is seen following postmyocardial systolic augmentation; and whereas survival period in all of these control animals thus prepared has been 2 hours, survival time here has been significantly increased by augmented circulation (Fig. 3).

*Discussion and applications.* While previous attempts have been made to accomplish augmentation of the circulation by withdrawal of blood during systole and reinjection during diastole, difficulties have been encountered in 2 ways: (1) size of the pump must be adequate effectively to change blood pressure; (2) timing of reinjection must be such as effectively to accomplish reinjection during the short period of ventricular diastole.

What are the untoward effects of incoordinated phasing? Obviously, if the reinjection phase of the augmenting ventricle occurs synchronously with systole of the true ventricle, the resultant force will oppose the rate of ejection of the true ventricle, and thus increase its work load. The work performed by the left ventricle is directly proportional to the stroke volume times the resistance against which the stroke volume is ejected; thus, if the resistance is decreased, the work of the left ventricular myocardium is proportionately decreased.

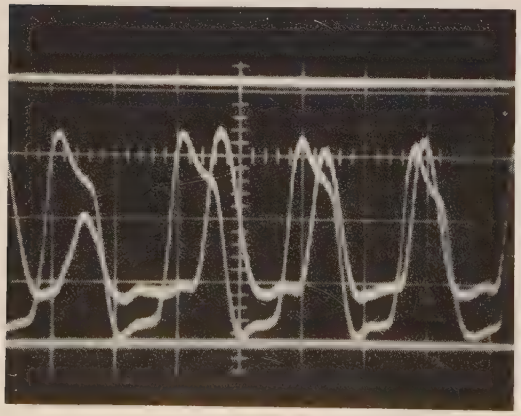

FIG. 3.

The method which we have developed allows the work of the myocardium to be reduced to a minimum so that blood pressure and flow may be maintained at a high level in the greater circulation. A device has been designed which functions in 2 stages. The first stage maintains adequate diastolic pressure in the systemic circulation; the second stage removes a great percentage of the work load of the left ventricle.

*Summary.* 1. Equipment has been developed which removes blood from the circulation during cardiac systole, thereby reducing intraventricular systolic blood pressure and reducing the work of systolic ejection of the heart. This equipment reinjects the blood during ventricular diastole, thereby increasing diastolic blood pressure and providing coronary blood flow. 2. An electronic circuit has been developed which enables the phasing of the augmenting systole to be accurately placed in relationship to the QRS complex of the EKG. Thus, timing of reinjection may be placed at any desired interval after the "R" wave of the EKG. 3. An animal preparation (dog) has been developed which combines a cardiac lesion produced by administration of norepinephrine combined with removal of one-third of the circulating blood volume and which is uniformly fatal. This procedure uniformly results in death of the control animal within an average period of 2 hours. Such animals uniformly develop dilated hearts and marked hypotension. 4. Survival time of animals thus prepared has been uniformly increased by application of the procedure of postsystolic myocardial augmentation. 5. The dilated heart and hypotensive state of the standardized animal in the preparation described above is reversed, and normal circulatory dynamics are approximated by application of this technic.

Received April 10, 1961. P.S.E.B.M., 1961, v107.

# Catecholamine Levels of Mouse Sympathetic Ganglia Following Hypertrophy Produced by Salivary Nerve-Growth Factor. (26720)

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A protein fraction isolated from mouse submaxillary gland has been shown by Levi-Montalcini and Cohen to have a powerful stimulating effect on the growth of peripheral ganglion cells, both in tissue culture as well as *in situ*(1-3). Daily injection of this "nerve-growth factor" (NGF) in newborn mammals produced as much as 6-fold enlargement of the sympathetic ganglia, while other types of neural tissue (as well as all non-neural organs) showed no morphological changes. Protein, RNA and DNA content of the ganglia increased 6-fold, 3-fold and 2-fold, respectively. In spite of this dramatic structural alteration in the autonomic nervous system, no functional changes have been detected. The purpose of this paper is to confirm the NGF effects reported by Levi-Montalcini and Cohen, and to show that the hypertrophy of the ganglia involves proportionate increase in their catecholamine content.

*Materials and methods.* The NGF component of mouse submaxillary glands was purified using a procedure similar to that described by Cohen(3), omitting, however, the DEAE- and one of the CM-cellulose column extractions.* The resulting fractions were assayed in tissue cultures of chick embryo spinal ganglia(4) and showed potencies of 7-160 units/$\mu$g dry weight (1 unit of NGF being defined as that quantity per ml of culture medium which produces a "3+" neuritic outgrowth after 1 day of incubation). Newborn mice were given daily subcutaneous injections of 500-1000 NGF units/g body weight. The fractions were generally lyophilized for long-term storage, and dissolved in physiological salt solution for injections at 0.05 ml/g body weight. Litters were restricted to 8 mice—4 treated and 4 controls (the latter receiving equal volumes of salt solution alone). The mice were sacrificed one day after 13-24 daily injections. The stellate and superior cervical ganglia were dissected out of each mouse, weighed in groups of 8-12 (consisting of equal numbers of the 2 types of ganglia). The resulting 4 to 8 mg of tissue was submerged in 1.0 ml of 0.01 N HCl in conical ground-glass homogenizer tubes. After homogenizing and washing the tube with 1.0 ml of 0.01 N HCl, the combined acid phase plus 2 g NaCl was extracted with 25 ml n-butanol by shaking 5 minutes. The norepinephrine (NE) was extracted into 1 ml of 0.01 N HCl from 20 ml of the n-butanol phase plus 20 ml n-heptane, using the same extraction conditions. For analysis of NE and epinephrine (EP), 16 ganglia were extracted by the same procedure into a final volume of 1.6 ml acid. Standard solutions of the amine in 0.01 N HCl and an acid blank were run with each set of samples. Aliquots of 0.6 ml of the acid phase were used to determine NE or EP by the method of Shore and Olin(5), using an Aminco spectrophotofluorometer. Except for changes in volume and in homogenization details the same procedure was used for the other tissues.

*Results.* After about 2 weeks of NGF injections, the sympathetic ganglia of the mice were found to be enlarged as much as 5-fold (Fig. 1 cf. 1, Fig. 6-7). Catecholamine levels were determined on a series of litters involving smaller, but still clearcut, hypertrophy (Table I). The wet weights of the NGF-treated ganglia ranged from 1.6 to 3.2 times control levels, but these values included variable amounts of connective tissue. The ratios are likely to be low in view of the greater difficulty in "cleaning" the smaller, untreated ganglia. The increase in catecholamine content per ganglion ranged from 1.7- to 4.1-fold, this ratio being greater in each of the 7 litters than the ratio of tissue mass increase. The NE *concentration* of NGF-hypertrophied ganglia has therefore been maintained (and possibly increased) during these dynamic conditions of growth. In litter 5, epinephrine analyses were made in addition to those for norepinephrine. In contrast to the 3-fold increase in NE per ganglion to 19 mμg, the EP content remained at the barely detectable level of about 1 mμg. On the basis of the specificity of this analytical procedure with reference to these and closely related amines(6), it seems clear that the observed NGF-evoked increase in ganglion catecholamine consists predominantly of NE. It should be pointed out that the data from the NGF-treated mice have been presented by litters in order to emphasize the correspondence between catecholamine increase and hypertrophy of these ganglia under a variety of experimental treatments. On the other hand, the weights and catecholamine content of the control ganglia from all 7 litters have been pooled since no significant differences as a function of body weight or age were detected. It is of interest, for example, that increases in ganglion mass and NE content were still substantial even when toxic side effects of relatively im-

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* Purification was carried out by Otto Walasek (Biochemistry Dept.) and by Ronald Gendrich and Dr. Wilfred White (Biochemical Development Dept.).

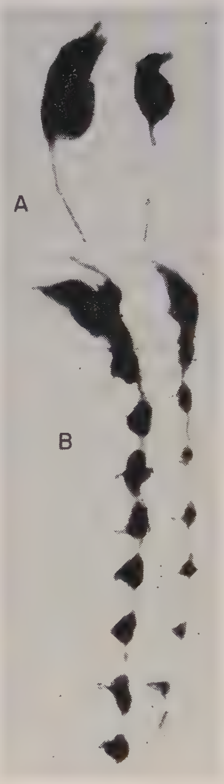

FIG. 1. Enlarged sympathetic ganglia (A: superior cervical; B: stellate and thoracic) of a 15-day NGF-treated mouse (left) in comparison with a 15-day control ganglion chain (right).

TABLE I. Catecholamine Levels of Sympathetic Ganglia from Control and NGF-Treated Mice.

| | Litter | | | | | | |
|---|---|---|---|---|---|---|---|
| | Treated | | | | | | Control |
| | 1 | 2 | 3, 4 | 5 | 6 | 7 | |
| NGF potency (units/μg protein) | 7 | 7 | 16 | 16 | 33 | 160 | — |
| No. of daily injections (500-1000 units/g body wt) | 16 | 14‡ | 13 | 24 | 13 | 22 | — |
| Avg body wt (g) at detn. (1 day after last inj.) | 3.7<br>(8.4)† | 6.5<br>(7.0) | 7.7<br>(8.8) | 14<br>(16) | 10<br>(10) | 14<br>(14) | — |
| No. of ganglia used | 8<br>(12)† | 8<br>(10) | 24<br>(23) | 15<br>(16) | 12 | 16<br>(16) | 77 |
| Avg wet wt of ganglion (mg) | .56 | .70 | .85 | 1.1 | .63 | .67 | .34 ± .6§ |
| Wt of NGF ganglion/control wt | 1.6 | 2.1 | 2.5 | 3.2 | 1.8 | 2.0 | — |
| Avg NE content of ganglion (mμg*) | 7.9 | 12 | 13 | 19 | 10 | 14 | 4.6 ± .8§ |
| NE content of NGF gangl./control content | 1.7 | 2.6 | 2.8 | 4.1 | 2.2 | 3.0 | — |
| Avg NE conc. of gangl. (mμg/mg tissue) | 14 | 17 | 15 | 17 | 16 | 21 | 14 ±3 § |

* mμg = millimicrograms = $10^{-9}$ g.

† Figures in parentheses refer to controls for specific litters.

‡ Somatotrophic growth hormone (Armour) was inj. along with NGF in these mice (*ca.* 15 μg/g body wt).

§ Mean ± stand. dev.

pure NGF fractions drastically curtailed body growth, *e.g.*, litter 1(cf. 1-3).

NE concentrations were determined in several other organs in litters 2, 5 and 7. A ratio of these concentrations in treated with respect to control mice was obtained using pooled organs from 2 to 4 treated animals and an equal number of controls. The mean and standard deviation of 3 to 4 ratios per organ were as follows: submaxillary gland, 2.3 ± 0.6; heart, 1.9 ± 0.4; brain, 1.0 ± 0.1; adrenal gland, 0.9 ± 0.1; spleen, 0.9 ± 0.2; and liver, 0.8 ± 0.2.

*Discussion.* The data suggest that NGF-induced hypertrophy of sympathetic ganglia involves increase not only in total protein and nucleic acids, but also in molecules which play a special role in the normal functioning of these neurons. It will be of interest to determine whether this hypertrophy leads to significant physiological effects, both in the normal as well as the neurologically damaged organism.

Our observation of 2- to 5-fold enlargement of sympathetic ganglia following daily injections of newborn mice with 500-1000 NGF units/g body weight is in good agreement with the work of Levi-Montalcini and Cohen. They reported use of 300-670 units/g to produce 3- to 6-fold ganglion hypertrophy. Considering the variations in our salivary purification procedures and tissue culture NGF-potency interpretations, the above differences in required dosage are quite small.

The lack of agreement between degree of ganglion hypertrophy produced in some of our litters by apparently similar dosage (*e.g.*, litters 5 *vs* 7 in Table I), is not surprising in view of the limitations in tissue culture assay of the various NGF fractions(4) as well as biological variations in NGF sensitivity that may well occur. Serious quantitation of the dose-response relations must await availability of larger amounts of purified NGF.

Our value of 14 ± 3 $\mu$g NE/g of mouse sympathetic ganglion tissue agrees well with the concentrations reported by Lissák(7) for adult cat and rabbit superior cervical ganglia, *i.e.*, 16 and 14 $\mu$g/g respectively.

The approximately 2-fold increase in NE concentration of heart and submaxillary gland in our NGF-treated mice may reflect the "hyperneurotization of the viscera" determined by histological methods(1). Although the lack of NE increase in brain and adrenal gland of the treated mice is quite reasonable in view of Levi-Montalcini's morphological observations(2), analysis of the spleen and liver results will require further investigation.

The recent report by Selye *et al.*(8) that isopropyl noradrenaline (isoproterenol) can evoke selective, 5-fold enlargement of (rat) salivary glands may be pertinent to analysis of the mechanism by which a (mouse) salivary gland component acts as a selective stimulant of growth and catecholamine content of sympathetic ganglia.

*Summary.* (1) Previous reports(1-3) of powerful growth-stimulating effects of a mouse submaxillary gland protein fraction (NGF) on certain peripheral ganglia have been confirmed. (2) Norepinephrine content of enlarged sympathetic ganglia in NGF-treated mice increased proportionate to tissue mass (3- to 4-fold), while epinephrine content remained barely detectable. (3) Norepinephrine concentrations of the heart and submaxillary gland increased about 2-fold in NGF-treated mice, while no significant increases were detected in brain, adrenal gland, spleen and liver.

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Received April 19, 1961. P.S.E.B.M., 1961, v107.

# Distribution of Copper and Molybdenum in Liver Centrifugal Fractions.*†
(26721)

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The toxicity of molybdenum has been recently reviewed(1,2). Feeding excess molybdenum to rats resulted in elevated liver levels of molybdenum and copper(3). Addition of inorganic sulfate to the diet reduced these elevated liver molybdenum and copper levels. The distribution of molybdenum in liver and kidney centrifugal fractions of chicks fed a tracer dose of $Mo^{99}$ has been determined by Teekell and Lyke(4). Mills§ has determined the distribution of copper and molybdenum in liver centrifugal fractions. In other studies the distribution of copper as influenced by hypophysectomy and growth hormone was ascertained(5).

Despite the work done on molybdenum toxicity, there is little information concerning the mechanism of toxicity or the effect of molybdenum on copper metabolism. It has been suggested that molybdenum may form a complex with copper in the tissues which renders the copper unavailable(6). Molybdenum may also directly interfere in enzyme processes(7,8,9). Although in many instances molybdenum toxicity closely resembles a copper deficiency that can be overcome by dietary copper additions, attempts to demonstrate a copper deficiency at the enzyme level have often been unsuccessful(9, 10). However, the activity of calf heart cytochrome oxidase(11) and sheep skin tyrosine oxidase(7) may be reduced with molybdenum feeding.

The results reported here were obtained from studies to determine the influence of dietary molybdenum and sulfate upon distribution of copper and molybdenum in particular fractions isolated from the livers of rats and perhaps furnish information about the mechanism of molybdenum toxicity.

*Methods.* The rats used in these studies (weanling Sprague-Dawley strain males) were individually housed in stainless steel wire floored cages and were provided food and water *ad libitum*. The basal diet was essentially the same as described elsewhere (3) and contained in per cent: sucrose 81.5, vitamin-free casein (N. B. Co.) 10.0, Wesson oil 5.0, mineral mixture 2.5, and vitamin mixture 1.0. Copper at the level of 1.6 ppm was added as the sulfate to bring total copper, as analyzed, to approximately 2.5 ppm. Four levels of molybdenum and 3 levels of sulfate were added to the diets of the 6 treatment groups as indicated in Table I. After feeding the experimental diets for 6 weeks, the rats were sacrificed by decapitation and the livers removed. Approximately half of each liver was dried, then analyzed for copper and molybdenum. The other portion of the fresh liver was immediately placed on ice and homogenized with 9 volumes of cold 0.25 M sucrose for 2.5 minutes. The homogenate was centrifuged at 700 × g for 10 minutes, the supernatant removed, the nuclei and debris precipitate resuspended in approximately 5 ml of ice cold 0.25 M sucrose and recentrifuged at 700 × g for 10 minutes. The 2 supernatants were combined and centrifuged at 5,000 × g for 10 minutes. As with the nuclei and debris fraction, the sedimented mitochondria were resuspended in sucrose and recentrifuged at 5,000 × g for 10 minutes. The supernatants were combined and designated as the supernatant fraction in the case where no further fractionation was done. In a second study a microsomal fraction was prepared by centrifuging the supernatant, from which the mitochondria had been isolated, at 75,000 × g for one hour. The microsomal pellet was washed but not resuspended. A standard wet ashing method, using nitric and perchloric acids, was used to

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* Preliminary results were presented in *Fed. Proc.*, 1961, v20, 296.

† Supported in part by a grant from Nutrition Foundation, Inc., New York.

‡ N.S.F. Fellow (Coop.).

§ Personal communication, Dr. C. F. Mills, Rowett Institute, Scotland.

TABLE I. Distribution of Cu and Mo in 3 Liver Centrifugal Fractions (% of Total, 5 Rats/Treatment).

| Diet additions | | | | | | | |
|---|---|---|---|---|---|---|---|
| ppm Mo* | 0 | 200 | 300 | 400 | 500 | 500 | 500 |
| ppm $SO_4$† | 0 | 0 | 0 | 0 | 400 | 750 | 1500 |
| Wt gain (g)‡ | 174 ±26 | 97 ±33 | 65 ±23 | 38 ±11 | 38 ±13 | 72 ±19 | 124 ±29 |
| | | | | Cu | | | |
| Nuclei and debris | 14 ± 3.7 | 25 ± 4.5 | 23 ± 7.2 | 28 ± 7.4 | 30 ± 4.7 | 34 ± 8.5 | 24 ± 6.4 |
| Mitochondria | 20 ± 4.7 | 28 ± 3.4 | 27 ± 2.4 | 28 ± 1.9 | 29 ± 6.2 | 28 ± 8.5 | 27 ± 4.5 |
| Supernatant | 66 ± 5.4 | 46 ± 4.6 | 50 ± 2.5 | 44 ± 8.3 | 42 ± 9.2 | 38 ± 3.7 | 48 ± 8.3 |
| Total (μg)§ | 31 ± 3.8 | 36 ± 6.1 | 27 ± 3.5 | 27 ± 5.3 | 25 ± 8.1 | 33 ± 2.2 | 34 ± 9.5 |
| Conc. (ppm)‖ | 10 ± 2.8 | 15 ± 4.4 | 18 ± 3.0 | 29 ±11 | 20 ± 8.0 | 19 ± 4.0 | 14 ± 3.6 |
| | | | | Mo | | | |
| Nuclei and debris | — | 10 ± 2.9 | 8 ± 3.3 | 13 ± 4.3 | 13 ± 3.3 | 17 ± 3.1 | 10 ± 3.8 |
| Mitochondria | — | 15 ± 1.0 | 14 ± 4.1 | 16 ± 1.9 | 15 ± 3.5 | 17 ± 4.0 | 16 ± 5.2 |
| Supernatant | — | 76 ± 3.4 | 78 ± 6.7 | 72 ± 5.8 | 72 ± 6.4 | 66 ± 3.5 | 74 ± 7.5 |
| Total (μg)§ | 2 ± .4 | 40 ±13 | 45 ±13 | 31 ± 8.6 | 42 ±17 | 36 ±10 | 43 ±11 |
| Conc. (ppm)‖ | .7± .2 | 20 ± 5.5 | 33 ±10 | 35 ±14 | 35 ±13 | 21 ± 3.2 | 18 ± 6.6 |

* Mo added to diet as $Na_2MoO_4 \cdot 2H_2O$.

† $SO_4$ added to diet as an equimolar mixture $K_2SO_4 : Na_2SO_4$.

‡ 6-wk gain in body wt.

§ Calculated from total liver wt and analysis of an aliquot.

‖ Dry wt basis.

digest the samples. Copper was determined by the carbamate method of the A.O.A.C. (12) and molybdenum by the thiocyanate method of Evans *et al.*(13).

*Results.* The feeding of up to 400 ppm of molybdenum severely limited rat growth and increased the liver levels of copper and molybdenum (Table I). Additions of inorganic sulfate improved the growth of molybdenum-fed rats and reversed the tendency toward increased concentration of liver copper and molybdenum. Distributions of copper and molybdenum in the centrifugal fractions are also given in Table I. An average of 66% of the total copper was present in the supernatant of the livers of the rats fed the control diet (–Mo) while in the molybdenum-fed rats, supernatant copper was approximately 45% of the total. This difference was statistically significant. The percentage of the total copper in the nuclei and debris and mitochondria fractions prepared from the livers of rats fed molybdenum was increased over that found in the same fractions prepared from livers of rats fed the control diet. When molybdenum was fed, it was found predominately in the supernatant. There was no significant effect of increasing dietary molybdenum above the lowest level added upon distribution of molybdenum or copper. It appeared that dietary sulfate had no significant effect on distribution of copper and molybdenum in the particulate fractions of liver.

To determine the amount of copper and molybdenum associated with the microsomes as a result of molybdenum feeding a second experiment was initiated. Six rats were fed the basal diet and 6 were fed basal plus 400 ppm molybdenum for 6 weeks. From the livers of rats in this trial, a microsomal fraction was prepared in addition to the other particulate fractions. The distribution results were qualitatively similar to those reported in Table I. In addition, the copper associated with the microsomal fraction represented about 10% of the total and did not appear to be altered when molybdenum was included in the diet. Likewise, the amount of molybdenum in the microsomal fraction was approximately 10% of the total.

*Discussion.* The largest amount of copper and molybdenum on an absolute and molar basis was not found in the same liver fractions, thus it appears unlikely that molybdenum causes copper concentration to rise by simply forming a copper-molybdenum complex. The increase in copper concentration in the particulate fractions may constitute a compensatory mechanism, namely, an attempt to overcome a copper deficiency induced by a molybdenum-block of a copper dependent metabolic reaction. Perhaps the failure to demonstrate a copper deficiency at the enzyme level is due to this extreme compensation.

The copper appeared to be associated with the particulate fractions of liver containing a high concentration of protein. The converse appears to be true for molybdenum. This suggests that the copper may be bound to protein as it is in blood.

*Summary.* In the liver of control rats (–Mo) the copper appeared to be concentrated ($> 55\%$) in the supernatant fraction (nuclei and debris $< 15\%$; mitochondria 20%; microsomes 10%), while in the liver from molybdenum-fed rats more copper was found in the nuclei and debris (27%) and mitochondria (27%) fractions and less in the supernatant ($<37\%$). The percentage of total copper in the microsomes appeared to be unaltered by dietary molybdenum. The molybdenum was usually found to be concentrated ($>60\%$) in the supernatant (nuclei and debris $< 15\%$; mitochondria 15%; microsomes 10%). The feeding of sulfate had little or no effect on distribution of copper or molybdenum.

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Received April 28, 1961. P.S.E.B.M., 1961, v107.

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## Normal and Experimental Involution of Rat Mammary Gland.* (26722)

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Previous studies in this laboratory have employed desoxyribonucleic acid (DNA) as a quantitative index of mammary gland proliferation during various experimental conditions(1,2,3,4) and during normal pregnancy and lactation in the rat and mouse(4,5,6,7). It was thought desirable to investigate reversion of DNA during normal involution of the rat mammary gland and to determine the effect of the ovarian hormones estrogen and progesterone upon this physiological process. These data will serve as a basic reference for further experiments on prevention of mammary gland involution.

*Materials and methods.* Data were obtained from primiparous Sprague-Dawley-Rolfsmeyer rats. Young were separated from dam on day 1 of lactation and mammary gland DNA was determined on days 5, 10, 14, and 20 after weaning. Experimental animals received subcutaneous injections of 1 μg E (estradiol benzoate) or 1 μg E + 3 mg P (progesterone) from day of weaning.

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* Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2294. Approved by Director. This investigation supported in part by grants from U.S.P.H.S. and Am. Cancer Soc.

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TABLE I. Total Mammary Gland DNA during Normal and Experimental Involution.

| Day of involution or lactation | No. of animals | Treatment | B.W. (g) mean | DFFT (mg) mean | DNA/mg (μg/mg DFFT) | Total DNA (mg/100 g) |
|---|---|---|---|---|---|---|
| Day 1 of lactation(5) | 11 | Normal | 245 | 584 | 35.6 ± .95* | 8.46 ± .77* |
| Day 20 of lactation(5) | 9 | " | 305 | 1210 | 28.3 ± .45 | 11.30 ±1.0 |
| Day 5 of involution | 27 | " | 258 | 494 | 32.2 ± 1.01 | 6.10 ± .47[2] |
| 10 | 19 | " | 266 | 347 | 30.1 ± 1.51 | 3.76 ± .25[4] |
| 14 | 9 | " | 277 | 389 | 30.2 ± 1.69 | 4.02 ± .19[5] |
| 20 | 11 | " | 279 | 471 | 21.4 ± .93 | 3.47 ± .25[6] |
| 5 | 21 | 1 μg E | 259 | 386 | 30.1 ± 1.05 | 4.48 ± .13[1,6] |
| 10 | 10 | " | 264 | 389 | 32.4 ± 1.23 | 4.95 ± .42[7] |
| 14 | 11 | " | 271 | 438 | 36.9 ± 1.11 | 5.88 ± .28 |
| 20 | 15 | " | 291 | 539 | 32.1 ± 1.3 | 5.84 ± .2 |
| 5 | 10 | 1 μg E + 3 μg P | 273 | 482 | 33.4 ± 2.15 | 5.64 ± .30[3] |
| 10 | 10 | *Idem* | 270 | 566 | 35.3 ± 1.18 | 7.46 ± .59 |
| 14 | 10 | " | 284 | 517 | 33.6 ± 1.66 | 6.18 ± .25 |
| 20 | 19 | " | 284 | 725 | 27.6 ± 1.17 | 6.91 ± .35 |

[1] Significant to day 1 of lactation at .1% level. [2] Significant to day 1 of lactation at 2% level.
[3] Significant to day 1 of lactation at 1% level. [4] Significant to day 5 of involution at .1% level.
[5] Significant to day 5 of involution at 5% level. [6] Significant to day 5 of involution at 1% level.
[7] Significant to day 5 of involution at 2% level.

* Mean ± S.E.

Six abdominal-inguinal mammary glands were removed from each rat and DNA determined as previously described(6).

Percentages were calculated after subtracting the base of 2.83 mg DNA/100 g from experimental values(5).

*Results.* Mammary gland DNA was reported as 8.46 mg/100 g BW (body weight) on day 1 of lactation(5). During course of normal involution, DNA decreased to 6.10 mg/100 g BW by day 5 of involution and at days 10, 14, and 20, DNA values were 3.76, 4.02, and 3.47 mg (Table I). DNA/100 g BW of experimental animals which received E alone was 4.48, 4.95, 5.88, 5.84, and with E + P, values were 5.64, 7.46, 6.18, and 6.91 at days 5, 10, 14 and 20, respectively, of involution. Significant differences exist between day 1 of lactation, and all days of normal and early experimental involution. During normal involution days 10 ($P<.001$), 14 ($P<.05$), and 20 ($P<.01$) were significantly reduced in relation to day 5 of involution, but marked differences were not observed between other days. At day 5 of involution, E group was markedly decreased below both normal ($P<.01$) and E + P groups ($P<.001$). By day 10, E ($P<.02$) and E + P ($P<.01$) values were greater than observed at same period of normal involution. This was evident throughout the remainder of experimental period. E + P DNA values were also consistently greater than obtained with E alone.

*Discussion.* Early morphological studies on rat mammary glands indicated that by day 5 of involution, only a small number of alveoli remained, the majority of glandular tissue being replaced by adipose tissue. Mammary glands resembled those of a normal virgin animal by day 9 of involution(8).

Present data agree with earlier studies. DNA decreased approximately 42% by day 5 of involution when compared to day 1 of lactation. By day 10, an 85% decrease is evident which then remains essentially constant throughout the remainder of the period. These values are comparable to those of normal virgin animals or ovariectomized controls(3). Preliminary data indicate when young are weaned after a normal lactation period (20 days), mammary glands involute to essentially the same values by days 10 and 20. DNA values for day 10 were 3.84 and day 20, 2.98 mg/100 g BW. This may be due to a faster rate of involution since

DNA values during lactation are significantly higher than observed at day 1 of lactation.

E was found to augment involution during the initial period after weaning. DNA was decreased 71% from day 1 of lactation, and 50% below the normal controls. By day 10, growth effects of E were evident with a 79% increase above control levels by day 20.

E + P did not prevent the initial decrease of DNA. Involution progressed at same rate as under normal conditions. P in combination with E appears to prevent more rapid decrease in DNA as found when E was injected alone. P may have a "masking" effect upon E, since it has been reported to retard involution at 1 mg/day for 6 days(8). E + P growth effects were evident by day 10, with an 82% increase over normal values by day 20.

Ovarian hormones may not retard involution to any significant degree, because DNA values determined at day 5 of involution were as low or lower than observed during normal period. Higher values observed with hormones during later stages of present experiment may be due entirely to growth promoting activity of these hormones upon the mammary gland.

*Summary.* With DNA as a measure of mammary gland involution, a 42% decrease in DNA between day 1 of lactation and day 5 of involution was noted. By day 10, DNA decreased 85% and remained essentially constant throughout the remainder of the period. E alone appeared to augment retrogression of DNA during initial days of involution, but with E + P this effect was not apparent. With both E and E + P, mammary gland DNA was increased during the later days of experimental period.

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Received May 1, 1961. P.S.E.B.M., 1961, v107.

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## Biologically Active Peptides in *Physalia* Toxin.* (26723)

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Several previous communications from this laboratory have detailed the activity of various kinds of crude *Physalia* toxin preparations(1,2,3). Recently whole toxin has been fractionated chromatographically into a series of peptides. Even after this rigorous treatment, some biological activity persists. This communication presents the results of tests for biological activity of crude toxin on various microorganisms and protozoa, and describes the distribution of toxicity among the component peptides of the crude toxin of *Physalia*.

*Materials and methods.* Surviving nematocysts were separated from tentacular tissue by controlled autolysis, and were homogenized to liberate their toxic contents as described by Lane and Dodge(1). Nematocysts were homogenized for 20 to 30 minutes in an all-glass homogenizer. The residue was diluted with 2 to 3 ml of distilled water and centrifuged for 20 minutes at 2°C and 10,000 rpm. The residue was resuspended in a

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* Aided by a grant from Nat. Inst. Health and by a contract between Office of Naval Research and Univ. of Miami. Scientific Contribution from Inst. of Marine Science.

† Aided in part by the American Physiol. Soc.

minimum of water and recentrifuged as before.

The supernatant solution was transferred quantitatively to tared 5-ml drying bulbs and lyophilized at 100-150 μ residual pressure. The drying bulbs were sealed without loss of glass while still evacuated. Six tared empty bulbs were evacuated to 125 μ residual pressure, sealed, cooled and re-weighed. The average weight loss (18.90 mg) provided a correction for calculation of total dry weight of toxin contained in each of the sealed drying bulbs. Lyophilized toxin was stored in evacuated drying bulbs at –10°C.

The yeasts and bacteria used for the auxanographic tests were isolated from Biscayne Bay. *Paramecium caudatum* and *Tetrahymena gellii* were obtained from laboratory cultures and *Uca pugilator* were collected on tidal flats near the Institute of Marine Science.

Fourteen different species of yeasts and 10 species of marine bacteria were exposed in duplicate to 4 different concentrations of *Physalia toxin*. Actively growing cultures of microorganisms were concentrated by centrifuging. The residue was adjusted with sterile distilled water to 20% transmission at 450 mμ. This "constant" suspension was diluted 1 to 20 with agar nutrient medium and poured into Petri dishes. Five sterile, antibiotic testing discs were arranged in a standard pattern on each plate. The center disc received 0.04 ml of sterile, distilled water. The 4 remaining discs each received 0.04 ml of 1.0, 10.0, 100.0, and 1000.0 p.p.m. of toxin solution. The plates were incubated at 25°C for 48 hours, then examined for the presence of zones of inhibition around each of the discs.

The effects of *Physalia* toxin on ciliate protozoa were assayed as follows: 100 living organisms of either *Paramecium caudatum* or *Tetrahymena gellii* were isolated from active cultures into 100 ml of growth medium. 1.0 ml of solution was added to each group of organisms containing the following toxin concentrations: 0.0, 10, 100, and 1000 p.p.m. Each concentration of toxin was replicated 4 times. Cultures were incubated at room temperature for 48 hours. They were then shaken well, and a 1 ml aliquot was removed from each. This was diluted with 5 ml of 10% formalin and brought to 50 ml with distilled water. All the organisms in 0.5 ml of this diluted fixed culture were counted. The 4 replicates of each toxin concentration were counted twice each by 3 individuals, and the results averaged and multiplied by 100 to determine number of organisms per culture. Preliminary to auxanographic tests or ciliate assays the activity of each toxin preparation was established by bio-assay on *Uca pugilator,* as previously described.

For chromatography of toxin fractions lyophilized toxin was diluted to 10,000 p.p.m. with distilled water and this diluted material was added as a stripe 5 inches long on Whatman #1 paper, 7½ by 18 inches in dimension. Chromatograms were run for 18 hours at room temperature in 80% aqueous n-propanol. The unstriped inch and a quarter at each edge of the papers received a single 10 μl spot of the same toxin dilution. The finished papers were dried, these edge strips were cut off and developed in 0.2% ninhydrin dissolved in acetone to reveal the location of the peptides on the rest of the paper. The papers were then cut so as physically to separate each peptide fraction. The fractions were separately eluted at room temperature with 2 to 5 ml distilled water. The chromatographic eluates were collected in tared 30 ml serum bottles and lyophilized directly. After elution of the individual peptides each paper was shown to be ninhydrin negative. Individual peptides separated chromatographically as described have been eluted, lyophilized, hydrolyzed with 6 N HCl in evacuated sealed capsules at 100° for 12 hours. After removal of hydrochloric acid the hydrolysate was rechromatographed. Each peptide was shown to contain more than 2 amino acids. Further details of the amino acid composition of each of these peptides will be described later.

After lyophilization the fractions were weighed and then accurately redissolved in sea water in proportion to their original concentration in the whole toxin. Each fraction was assayed on a group of 10 *Uca pugilator* evenly divided as to sex and of approximately

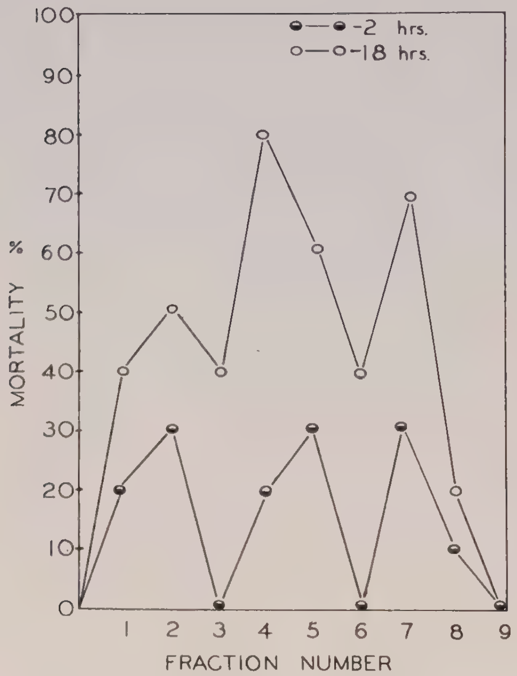

FIG. 1. Toxicity of chromatographic fractions of crude *Physalia* toxin. Ten *Uca pugilator* were inj. with each fraction and mortality recorded at 2 hr and again at 18 hr.

the same weight. 0.10 ml was injected through the arthrodial membrane at the base of the third walking leg. The results of the injection were evaluated after 2 hours and 18 hours.

*Results.* No toxin preparation exhibited activity in the auxanographic tests with a variety of marine microorganisms. Control colonies of *Tetrahymena gellii* and of *Paramecium caudatum* increased in number from 100 to 7800 ± 230 and 247 ± 26 respectively in 24 hours under the conditions of these tests. No significant change in growth rate occurred with 3 different concentrations of toxin. Fig. 1 shows that all component peptides of the crude toxin possess some toxicity for *Uca,* but peptides numbered 2, 4, 5, and 7, sequentially from the point of origin exhibited the highest biological activity.

*Discussion.* As has been previously reported from this laboratory, the toxin of *Physalia* is labile and it is easily destroyed by prolonged exposure to the effects of organic solvents. However, under the conditions of these experiments sufficient activity persisted in the chromatographic fractions to permit evaluation of one fraction against another. It is quite clear that the activity of the component peptides of the crude toxin of *Physalia* vary in biological activity as they have been shown to vary in chemical composition.

Each of these peptides is presently being studied in this laboratory and details of their chemical composition, component amino acids, and pharmacologic activity will be reported later.

*Summary and conclusions.* The crude toxin of *Physalia* nematocysts withstands lyophilization without significant loss of toxicity. *Physalia* toxin may be separated into component peptides by one-dimensional chromatography in 80% aqueous n-propanol. Each of the resultant peptides retains considerable toxicity for the fiddler crab, *Uca pugilator.* Crude toxin of *Physalia* nematocysts has no effect on growth of marine yeasts or marine bacteria. Toxin preparations have been shown to be without significant effect on growth or reproduction of the ciliate protozoans, *Paramecium caudatum* and *Tetrahymena gellii.*

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Received May 10, 1961. P.S.E.B.M., 1961, v107.

# Composition of Float Gases of *Physalia physalis*.* (26724)

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Recent communications(1,2) have described the float gases of *Physalia physalis* and have called attention to the presence of significant concentrations of carbon monoxide in this gas. Wittenberg(2) has suggested a possible route for biological synthesis of carbon monoxide from the amino acid serine. This communication describes a series of analyses of the float gases of *Physalia*, taken directly from living animals under nearly optimal conditions, and an experiment to show how the composition of the float gas is corrected after experimental modification.

*Materials and methods.* Living specimens of *P. physalis* were collected from the ocean beach on Key Biscayne, Fla., on days when the winds were favorable during the period of their seasonal abundance(3). It should be emphasized that all animals for this study were taken from outside the surf line before they had been stranded on shore and were transported directly to the laboratory with a minimum of handling. Gas was aspirated into special gas-sample bottles immediately upon arrival at the laboratory and stored under slight positive pressure in the same bottles. Gas samples were analyzed in the Scholander ½cc Gas Analyzer. A series of analyses for oxygen and carbon dioxide were made using the separate hydrosulfite-anthraquinone sulfonate and KOH absorbents described by Scholander(4). Since carbon dioxide was negligible in float gases, these absorbents were replaced by alkaline pyrogallate to absorb both oxygen and carbon dioxide together. Carbon monoxide was absorbed by Scholander's modification of Winkler's solution(5). The sample was transferred without contamination to the analyzer and allowed to equilibrate. The oxygen and carbon dioxide were absorbed by alkaline pyrogallate. The reaction chamber was then washed twice with acid-rinsing solution and rinsed one or more times with distilled water. Carbon monoxide was determined by introducing another sample of the same gas and absorbing the carbon monoxide, oxygen, and carbon dioxide with Winkler's solution, and subtracting from this value the percentage of oxygen and carbon dioxide which had already been determined. At least 2 complete analyses were made of each gas sample, and unless the results agreed within 0.1% the analyses were discarded and the sample analyzed again. After analysis of carbon monoxide the apparatus was again washed and rinsed completely in preparation for analysis of the next sample.

To demonstrate the capabilities of the gas gland of *Physalia*, one vigorous mature specimen was partially deflated by withdrawing gas from the pneumocyst and replacing it with pure carbon monoxide. The animal was then maintained in aquarium of circulating aerated sea water. A floating polyethylene ring prevented the specimen from adhering to the wall of the aquarium. This animal remained in good condition during the experiment.

Pressure within the float of mature vigorous specimens was measured by inserting a cannula connected with an inclined water manometer directly into the float cavity.

Certain specimens were partially deflated and subsequently observed for periods up to 36 hours. None was observed to reinflate.

In other experiments it was thought advisable to replace the gases of the float completely. To this end the pneumocyst was flushed 3 times with tank carbon monoxide. Although great care was exercised to avoid over-inflation no animal subjected to this treatment survived 24 hours.

*Results and discussion.* Table I describes the composition of the float gas in 12 individual *Physalia*. The composition varies considerably with various functional states of the animal. Carbon monoxide was the most

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* These studies were supported in part by Contract between Office of Naval Research and Univ. of Miami. Scientific contribution from Inst. of Marine Science.

TABLE I. Composition of Float Gas of *Physalia*.

| Sample | % $O_2$ | % CO | % N |
|---|---|---|---|
| 1 | 16.976 | 6.074 | 76.950 |
| 2 | 16.808 | 5.412 | 77.780 |
| 3 | 17.563 | 2.620 | 79.817 |
| 4 | 16.277 | 2.660 | 81.063 |
| 5 | 18.749 | 1.285 | 79.967 |
| 6 | 17.563 | 2.626 | 79.966 |
| 7 | 16.277 | 2.660 | 79.811 |
| 8 | 19.320 | .712 | 79.968 |
| 9 | 20.775 | .211 | 79.014 |
| 10 | 17.437 | 3.371 | 79.192 |
| 11 | 14.723 | 5.552 | 79.725 |
| 12 | 20.893 | 1.246 | 77.861 |
| Avg | 17.78 | 2.89 | 79.26 |

variable of the float gases. This observation lends support to the suggestion that the gas originally secreted by the gas gland is pure carbon monoxide. The structure of the float appears to minimize exchange between its contained gas and the atmosphere. It should be recalled that this apparently simple structure contains 2 mesogleal layers, 2 ectodermal layers, 2 endodermal layers, and an extension of the gastrovascular cavity, through which fluid circulates, and that it is lined with a chitinous cuticle which must further impede gas exchange. These structural conditions suggest that diffusional exchange between gases of the float and the ambient air must be minimal.

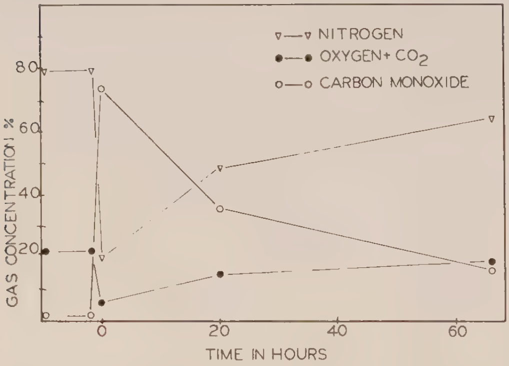

FIG. 1. Composition of *Physalia* float gases during a typical flushing experiment. At 0 hr approximately one-third of total gas volume was replaced by carbon monoxide.

Pressure within the float, measured directly, was from 5 to 10 mm Hg above atmospheric. It is unlikely that this is sufficient to establish a significant outward diffusion gradient. Since the partial pressures of component gases within the float normally equal or exceed those in ambient air, significant inward diffusion, postulated by Wittenberg(*2*) is also unlikely.

*Summary and conclusion.* The float gases of *Physalia* contain carbon monoxide. The amount varied from 0.21% to 6.07% of the total. It is suggested that variations in carbon monoxide content may reflect differing functional states in the organism. The composition of the gas in the pneumocyst may change qualitatively and quantitatively in 24 hours. When the float was inflated with pure carbon monoxide this gas was diluted approximately 4-fold during the following 66 hours. During this same period the concentration of oxygen and carbon dioxide increased about the same extent. Concentration of nitrogen in the float gas increased 3-fold during this same period. These changes in composition of float gases could result either from active secretion from the gas gland or from passive diffusion through the wall of the pneumocyst. Structural considerations make this latter possibility considerably more remote than active secretion by the gas gland.

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Received May 10, 1961. P.S.E.B.M., 1961, v107.

# Effect of Cephalosporin C and Various Penicillin Derivatives on Staphylococcal Penicillinase and Penicillinase-Producing Staphylococci.* (26725)

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Cephalosporin C, an antibiotic which is chemically related to, but not identical with penicillin(1,2), has been shown to be active against penicillin-resistant staphylococci(3) and insensitive to the action of purified *Bacillus cereus* penicillinase(4). The unavailability of this compound prevented verification and extension of these reports by other laboratories. With cephalosporin C made available to us through the courtesy of Squibb Institute for Medical Research, we undertook a series of investigations to compare cephalosporin C with penicillin G and a variety of penicillin derivatives as to their effect upon penicillinase-producing staphylococci and staphylococcal penicillinase.

*Materials and methods.* A number of strains of staphylococci isolated from clinical infections were available. For comparative purposes, strains 46 S (sensitive to penicillin) and 46 R (penicillinase-producing) which were genetically related were frequently employed. Susceptibility to the various antibiotics was determined by means of standard 2-fold tube dilution methods. Penicillinase activity was assayed by means of the manometric method adapted for use with staphylococcal cells(5) from the original method of Henry and Housewright(6). Induction of staphylococcal penicillinase synthesis was accomplished by the methods described by Geronimus and Cohen(7). Cephalosporin C, phenoxyethylpenicillin, and 6-aminopenicillanic acid were provided by Dr. Ralph E. Bennett of Squibb Inst. for Med. Res., dimethoxyphenylpenicillin (6-(2,6-dimethoxybenzamido)penicillanic acid) by Bristol Laboratories, and penicillin G by Charles Pfizer and Co. Other reagents and chemicals were obtained from the usual commercial sources.

*Results and discussion. Relative effectiveness of cephalosporin C against penicillin-resistant and penicillin-sensitive staphylococci.* Table I shows that cephalosporin C is equally effective against both penicillin-resistant and penicillin-sensitive strains of staphylococci. Penicillin G, which inhibits sensitive strains at concentrations of less than 0.15 μg/ml, is ineffective against some resistant strains at concentrations in excess of 300 μg/ml. Cephalosporin C, while not nearly so effective as penicillin G against sensitive strains, is more effective than penicillin G against penicillin-resistant strains when heavy inocula are employed. As shown for strain 604 R, with penicillin G minimal inhibitory concentration increases to a considerable extent with increased inoculum size, while with cephalosporin C there is a change of only one tube in the dilution series.

*Effect of combinations of penicillin G and cephalosporin C against penicillin-resistant staphylococci.* Crawford and Abraham(3) provided evidence for synergistic action between cephalosporin C and penicillin G. To test this possibility, tube-dilution series of both penicillin G and cephalosporin C in

TABLE I. Relative Effectiveness of Penicillin G and Cephalosporin C against Penicillin-Sensitive and Penicillin-Resistant Staphylococci.

| Agent tested | Hr incubation | Minimal inhibitory conc. (μg/ml)<br>Strain<br>46 R* | <br><br>46 S* | <br><br>604 R† | <br><br>604 R‡ |
|---|---|---|---|---|---|
| Penicillin G | 18 | 2.4 | <.15 | 9.4 | >300 |
| | 24 | 18.8 | ” | 37.5 | ” |
| | 48 | 37.5 | ” | 150 | ” |
| Cephalosporin C | 18 | 125 | 62.5 | 125 | 125 |
| | 24 | ” | ” | ” | 250 |
| | 48 | ” | ” | ” | ” |

* R = resistant to penicillin G; S = sensitive to penicillin G.

† Inoculum for the series shown in first 3 columns was 0.1 ml of a 1:1000 dilution of the inoculum culture.

‡ Inoculum for the series shown in last column was 0.1 ml of a 1:10 dilution of the inoculum culture.

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* Supported in part by grants from Squibb Inst. for Med. Res. and Nat. Inst. Health, U.S.P.H.S.

TABLE II. Effect of Combinations of Penicillin G and Cephalosporin C against Penicillin-Resistant *Staphylococcus aureus* 46 R.

| Agent tested | Agent added | Conc. added | Minimal inhibitory conc. (μg/ml) 24 hr | 48 hr |
|---|---|---|---|---|
| Pen. G | None | — | 75 | >300 |
| " | Ceph. C | 100 | .3 | 4.7 |
| " | " | 50 | 4.7 | >300 |
| " | " | 10 | 4.7 | >300 |
| Ceph. C | None | — | 62.5 | 125 |
| " | Pen. G | 100 | .5 | 62.5 |
| " | " | 50 | .5 | " |
| " | " | 10 | 15.6 | " |

which one agent was diluted while the other agent was maintained at a constant level were compared at several concentrations of each of the agents. As shown in Table II, 24-hour readings might lead one to conclude that the 2 agents act synergistically. However, minimal inhibitory concentration after 48 hours is within one to 2 tubes of that observed with either agent alone up to the point where the combined concentration of the 2 agents is in excess of the minimal inhibitory concentration for one agent alone. Viability end points taken at the 24-hour interval confirmed the fact that 48-hour visible end points were the more reliable. It appears that slight alterations in the end-points obtained with combinations of the 2 antibiotics are more likely a reflection of relative concentration of penicillin and inoculum size rather than as a result of any clear-cut synergistic action between the 2 agents. Crawford and Abraham(3) also indicated that the combined effect was less obvious with heavier inocula.

TABLE III. Effect of Staphylococcal Penicillinase on Cephalosporin C and Various Penicillin Derivatives.

| Substrate ($6 \times 10^{-3}$ M) | μl $CO_2$/mg/30 min.* |
|---|---|
| Penicillin G | 794 |
| α-Phenoxyethylpenicillin | 750 |
| 6-Aminopenicillanic acid | (0) |
| Dimethoxyphenylpenicillin | (0) |
| Cephalosporin C | (0) |

* Using cells in which penicillinase activity had been induced with dimethoxyphenylpenicillin. Relative rates of hydrolysis of the various substrates were comparable with uninduced cells or following induction with other derivatives.

*Effect of staphylococcal penicillinase on cephalosporin C and various penicillin derivatives.* Penicillin G and phenoxyethyl-penicillin are hydrolyzed at approximately the same rates by staphylococcal penicillinase (Table III). Cephalosporin C, 6-amino-penicillanic acid, and dimethoxy-phenylpenicillin are acted upon slowly, if at all. Steinman(8) recently reported that 6-amino-penicillanic acid and dimethoxyphenylpenicillin are hydrolyzed at definite, but very low, rates by both *B. cereus* and *S. aureus* penicillinases. While rates of hydrolysis of these compounds are of such a low order of magnitude as to be of relatively little practical importance, it is probably incorrect to assume that they are not attacked by the enzyme. Under the conditions of this experiment, the absolute rates of hydrolysis of these compounds were not detectable.

TABLE IV. Effect of Cephalosporin C on Induction of Staphylococcal Penicillinase.

| Inducer | Conc. | μl $CO_2$/mg/30 min.* |
|---|---|---|
| None | — | 76 |
| Penicillin G | $1.78 \times 10^{-3}$ M | 360 |
| Dimethoxyphenyl-penicillin | $1.78 \times 10^{-5}$ M | 940 |
| " | $.36 \times 10^{-5}$ M | 1,070 |
| " | $1.78 \times 10^{-6}$ M | 967 |
| Cephalosporin C | $3.54 \times 10^{-4}$ M | 1,160 |
| " | $1.78 \times 10^{-4}$ M | 1,220 |
| " | $.89 \times 10^{-4}$ M | 1,015 |

* Using penicillin G ($6 \times 10^{-3}$ M) as substrate. The results shown represent induced levels observed using concentrations of the inducer near the optimal levels determined from preliminary experiments conducted over a much wider range of inducer concentrations.

*Cephalosporin C as an inducer of penicillinase synthesis.* Cephalosporin C was found to be an active inducer of penicillinase synthesis by staphylococcal cells. Table IV shows a comparison of penicillinase activity in cells induced with cephalosporin C, dimethoxyphenylpenicillin, and penicillin G. Although the induced levels may vary slightly from one experiment to another, cephalosporin C and dimethoxyphenylpenicillin appear to be approximately equal in their inducing ability and both compounds are definitely superior to penicillin G in this respect. Not shown are the effects of 6-amino-penicil-

TABLE V. Effect of Cephalosporin C on Staphylococcal Penicillinase Activity.

| Inducer | Substrate* | $\mu$l $CO_2$/mg/30 min. |
|---|---|---|
| None | Penicillin G | 43 |
| " | Cephalosporin C | (0) |
| " | Pen. G + Ceph. C | 31 |
| Pen. G | Penicillin G | 240 |
| " | Cephalosporin C | (0) |
| " | Pen. G + Ceph. C | 238 |
| " | " + 5 × Ceph. C | 181 |
| Ceph. C | Penicillin G | 871 |
| " | Cephalosporin C | (0) |
| " | Pen. G + Ceph. C | 833 |
| " | " + 5 × Ceph. C | 787 |

* Penicillin G and cephalosporin C were employed at $6 \times 10^{-3}$ M or at 5 times this concentration where indicated.

lanic acid and phenoxyethylpenicillin which are intermediate between penicillin G and cephalosporin C in their ability to induce penicillinase synthesis. While levels of penicillinase activity following induction with cephalosporin C and dimethoxyphenylpenicillin are usually comparable, there is a definite difference in the concentration required for optimal induction. Our results with staphylococci compare favorably with those of Pollock(9) who found cephalosporin C to be an active inducer of *B. cereus* penicillinase and of Steinman(8) showing that dimethoxyphenylpenicillin induced both *B. cereus* and *S. aureus* penicillinase synthesis.

*Effect of cephalosporin C on penicillinase activity.* Variation in concentration of cephalosporin C from zero to as high as 1:1 molar ratios of cephalosporin C to penicillin G failed to elicit significant changes in the rate of penicillinase activity (Table V). At 5:1 molar ratios of cephalosporin C to penicillin G slight decreases in activity were observed. Abraham and Newton(4) reported that cephalosporin C competitively inhibited the soluble penicillinase of *B. cereus*. The affinity of the enzyme for cephalosporin C was indicated to be only about one-third that for penicillin G as evidenced by the requirement for relatively high concentrations of cephalosporin C to achieve significant inhibition. To the extent that relatively high concentrations of cephalosporin C are also required to inhibit staphylococcal penicillinase, our results are consistent with those obtained using the soluble penicillinase from *B. cereus*. In other experiments, preincubation of staphylococcal cells with cephalosporin C for periods of as long as 4 hours prior to addition of substrate failed to produce significant change in rate of penicillinase activity.

*Summary and conclusions.* 1. Cephalosporin C is equally inhibitory to penicillin-resistant and penicillin-sensitive staphylococci. However, against sensitive strains it is not nearly so effective an antibiotic as penicillin G. 2. Cephalosporin C and penicillin G apparently do not act synergistically in inhibiting the growth of penicillin-resistant staphylococci. 3. Cephalosporin C, dimethoxyphenylpenicillin and 6-aminopenicillanic acid are hydrolyzed slowly, if at all, by staphylococcal penicillinase. 4. Cephalosporin C is an active inducer of penicillinase synthesis in staphylococci, being comparable to dimethoxyphenylpenicillin in this respect but superior to penicillin G. 5. Cephalosporin C, at relatively high concentrations, does not significantly inhibit staphylococcal penicillinase.

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Received May 12, 1961. P.S.E.B.M., 1961, v107.

# Transduction of Capacity to Produce Staphylococcal Penicillinase. (26726)

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Resistance of *Staphylococcus aureus* to penicillin seems to be due to 2 apparently different mechanisms. Resistance in staphylococci isolated from natural sources is related to capacity to produce the enzyme, penicillinase(1) while resistant staphylococci isolated *in vitro* by selection from large populations of cells do not produce this enzyme (2).

The present report concerns an investigation of phage-mediated transduction of capacity to produce penicillinase by *S. aureus*. Transduction was first described by Zinder and Lederberg who observed this type of genetic exchange in the genus *Salmonella*(3). The phenomenon was observed in the genus *Escherichia* by Morse(4); in the genus *Shigella* by Lenox(5); and in the genus *Staphylococcus* by Ritz(6) and later by Morse(7).

*Methods.* The strains of *S. aureus* employed were isolated during epidemiological studies of staphylococcal infections in nurseries at Ohio State University Hospital. The Standard International Typing Series of staphylococcal bacteriophages employed were received from Dr. John E. Blair. Cultures of *S. aureus* were maintained on brain heart infusion (BHI) agar slants containing 7.5% NaCl at 4°C as were suspensions of bacteriophages.

The bacteriophages were propagated on penicillinase producing strains of *S. aureus* using the method of Swanstrom and Adams (8). The agar medium (P and D) used for propagation and titration of bacteriophage was nutrient broth containing 0.25% $K_2HPO_4$ and 0.2% dextrose; the basal layer contained 1.5% agar, the seed layer 0.5% agar. P and D broth was used to harvest and to prepare dilutions of bacteriophages, and to wash and suspend bacterial cells. Maximum amounts of phage were obtained after incubation for 8 to 10 hr at 37°C. Phage lysates were rendered free of bacteria by passage through an 02 Selas filter.

The bacteriophage used in most experiments was phage 80, propagated on a strain of *S. aureus* designated as U-40. One ml of a lysate containing $2 \times 10^{10}$ plaque forming units of this phage, designated as 80/U-40, was mixed in a 15 × 150 mm culture tube with 1 ml of a broth suspension of the sensitive acceptor strain, C-72, which had been grown on a BHI agar slant for 18 hours at 37°C. The bacterial inoculum was adjusted to give a multiplicity of infection of 1.0. The suspension of phage and acceptor cells was shaken gently during incubation for one hour at 37°C. The transduction mixture then was centrifuged, the supernatant fluid discarded and the bacterial pellet resuspended in 2 ml of broth. After a second centrifugation, the pellet was resuspended in 0.9 ml of P and D broth. Using sterile glass rods, 0.05 ml samples of the suspension of cells were streaked onto the surface of BHI agar plates containing 0.12 unit of penicillin per ml of medium. Plates were incubated at 37°C for 18 hours before being scored for the number of penicillinase producing colonies.

*Results.* Exposure of a penicillin sensitive strain of *S. aureus* to a bacteriophage, propagated on a strain producing penicillinase, resulted in the appearance of colonies that had acquired the capacity to produce penicillinase. These colonies were readily detected by their large size and the satellite of penicillin-sensitive "persisters" in the area where penicillin had been inactivated by penicillinase. From 3 experiments, a total of 144 pure cultures were isolated from different colonies that had acquired the penicillinase marker. When tested for 12 distinctive physiological characteristics, the transduced clones were similar to the acceptor strain except for resistance to penicillin. The origin of the variants as a consequence of mutation and selection was refuted by the failure of massive inocula ($2 \times 10^{10}$ cells) of untreated suspensions of the acceptor strains to yield colonies on the selective medium. Furthermore, attempts to isolate penicillinase-producing variants of the acceptor strain by the gradient plate technic(9) were not successful.

The rate of transduction was 1-2 transduced cells per $10^7$ plaque forming units. Agitation of the suspension of phage and acceptor strain during the initial incubation period increased the yield of transduced cells 3 to 5-fold. Varying the time of the initial incubation period from 5 minutes to 2 hours revealed that one hour resulted in maximum numbers of transduced cells.

With the strains of *S. aureus* that were tested, 0.12 unit of penicillin per ml of medium yielded the largest number of transduced colonies that could be accurately scored. Concentrations only slightly greater than 0.12 unit caused a marked decrease in detectable transduced cells. Lower concentrations permitted the emergence of an undesirable background growth and the common 1st step mutants which were resistant but failed to produce penicillinase. Although low concentrations of penicillin were necessary for primary expression of transduced cells, subcultures grew readily in the presence of 250 units of penicillin per ml of medium.

When tested by the routine typing procedures, the donor strain U-40 was sensitive to lysis by phages 80 and 81 and mutants of phages 42B, 47C, 44A and 52. Three of the mutants, 42B, 47C, 52, were used successfully to transduce the penicillinase marker. The frequencies of transduction, using phages 42B and 47C, were considerably lower than those obtained with phages 52 and 80. Although phages 80 and 81 are considered to be closely related, all attempts to obtain transduction with phage 81 were unsuccessful.

Since the mutant phages 42B, 47C, and 52 and phage 80 were also highly lytic for recipient strain, C-72, transduced cells could not be detected on media on which lysis occurred. However, BHI or heart infusion agar (Difco) inhibited lysis by the above phage but not the transduction process and either medium could be employed for detection of transduced cells.

TABLE I. Bacteriophage Type of Acceptor Strains of *Staphylococcus aureus.*

| Strain | Bacteriophage type |
|---|---|
| C-72 | 29/52A/79/80 (42B, 47C, 52)* |
| W-26 | 29/79/80 (52) |
| CH-50 | 80 (52) |
| M-1 | 29/52A/55/79/80 (52) |

* (42B, 47C, 52) mutants of original phage.

Of 19 penicillin sensitive strains tested, 4 were capable of acting as recipients of the penicillinase marker following exposure to phage 52/U-40 or 80/U-40. The 4 acceptor strains were sensitive to lysis by bacteriophage in Group I. Most of the strains which failed to accept the penicillinase marker were sensitive to lysis by phage in Group III, and a few by phages in Groups I and III. The phage types of the acceptor strains are shown in Table I.

*Discussion.* The exchange of genetic material among strains of *S. aureus* by transduction opens the way for a genetic analysis of this important species. If transduction can be expanded among the staphylococci, an analysis using this technic will supply essential information on the problem of differentiation of species and strains. Furthermore, more precise and detailed information on the chemical nature, modes of action, and interrelationship of the toxins and enzymes of *S. aureus* coupled with an analysis at the genetic level could provide the ultimate solution to the problem of pathogenesis.

There has been widespread and rapid emergence of penicillinase-producing strains of *S. aureus* since the advent of penicillin therapy. However, the ultimate source of these strains is completely unknown. Fairbrother feels that the great number of strains which produce penicillinase represent original wild types that have persisted since the time when the natural habitat of the staphylococci was the soil(10). However, it is not unlikely that transfer of genetic material *via* bacteriophage occurs in a mixed population of staphylococci in nature and this could readily explain the divergence of penicillinase-producing strains that have been observed.

*Summary.* Transduction of capacity to

produce the enzyme penicillinase among strains of *S. aureus* was accomplished. Four of 19 sensitive strains were capable of accepting the penicillinase marker. The 4 acceptor strains were sensitive to lysis by phages in Group I. Four different phages were capable of participating in the transduction process.

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Received May 19, 1961. P.S.E.B.M., 1961, v107.

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# Embryonic Survival in Relation to Number and Size of Implantation Swellings in the Rabbit *Oryctolagus cuniculus*.* (26727)

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The mechanisms involved in implantation of blastocysts are different from those required for embryonic or fetal survival. The attachment of blastocysts depends on 3 principal mechanisms related to myometrial muscle, adhesions and invasion; these operate in sequence but with some overlap(1). Embryonic survival depends on other factors such as genetic makeup(2), age of female output of unknown substances in the dam's blood(3) and age of gametes at fertilization (4). Hormonal requirements of implantation are also different from those of embryonic survival(5). It has been shown experimentally that deficiency in progesterone can cause embryonic death at some stages of pregnancy(6).

In polytocous mammals, embryonic mortality is usually followed by reabsorption. With a very large number of implantations complete reabsorption of the litter during pregnancy is most common among younger females(7). In the rabbit, embryonic loss is distributed in a specific pattern; 7% immediately after implantation, 66% during mid-pregnancy and 23% during late pregnancy (8).

The purpose of this experiment is to examine embryonic survival in relation to number of implantations and diameter of uterine swelling at implantation sites. The ova transfer technic was used to obtain rabbits with different numbers of implantations within the physiologic range of the species (normal implantation number in the rabbit 5 to 15).

*Materials and methods.* A total of 56 pubertal and 49 adult rabbit does of New Zealand White, California, and crossbreds were used in this experiment. Average weights were 6.3 lb for pubertal does and 7.5 lb for adults. All rabbits were brought from one local breeder who used a mild degree of inbreeding. Prior to the experiments each doe was isolated for 16 days. Twelve fertile and vasectomized bucks were also available.

*Donors.* The pubertal does used as donors were superovulated by gonadotrophic hormones. Pregnant mare serum was used for the priming doses and HCG was used to induce ovulation. Soon after the ovulation-inducing injection, each donor was mated to

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* This work was supported by research grants from Nat. Science Foundation and the Population Council, Inc., N. Y.

Scientific Paper 2070, Washington Agric. Exp. Stations, Pullman, Project No. 1473.

TABLE I. Embryonic Survival† in Relation to Number of Implantations* in Both Uterine Horns.

| Items | | No. of implantations*/recipient<br>1–5 | 6–10 | 11–16 |
|---|---|---|---|---|
| No. of recipients | | 22 | 14 | 13 |
| No. of corpora lutea on both ovaries | Range | 6–14 | 7–16 | 10–15 |
| | Mean | 11.4 | 11.4 | 12.2 |
| Total No. of implantations* | | 73 | 107 | 160 |
| No. of implantations/recipients—Mean* | | 3.3 | 7.6 | 12.3 |
| No. of surviving embryos† | Range | 0–5 | 5–10 | 7–11 |
| | Mean | 2.0 | 5.6 | 9.2 |
| % embryo survival | Range | 0–100 | 25–100 | 47–100 |
| | Mean‡ | 56 | 74 | 76 |

* At 8 days post coitum.
† " 15 " " .
‡ Difference between the 3 means were not statistically significant.

2 fertile bucks. Forty to 48 hours post coitum, the donors were sacrificed by bleeding and the tubes were placed in sterilized Petri dishes. The ova were recovered by flushing the tubes with a few milliliters of sterilized physiological saline and were counted under a binocular microscope (×10), mixed in a watch glass and examined carefully (×45) for their normality. Healthy 8-32-blastomere ova were selected. The procedures were carried out inside a glass cabinet in a culture room kept at 25 to 28°C.

*Recipients.* Three groups of recipients were available; different numbers of fertilized ova were transferred to both uterine horns of the recipients. Recipients were mated to vasectomized bucks to induce pseudopregnancy. Recipients were anaesthetized with an intravenous injection of Nembutal (Abbott). Laparotomy was performed and 3 to 20 fertilized ova were transferred into each Fallopian tube. 25,000 I.U. of penicillin were sprayed in the abdomen near the site of incision.

The abdomen was closed in one layer using chromic gut (00) (Ethicon). Recipients were laparotomized from the midline at 8 days post coitum; number and diameter of implantation sites were recorded. At 15 days p.c., recipients were sacrificed and the reproductive tract was dissected. The number of corpora lutea on both ovaries was counted and viability of implantations was determined by dissecting the fetuses and examining the heart beat. The data included 340 implantations.

*Results and discussion.* The number of implantations expressed as a percentage of number of ova transferred averaged 83%. Nine recipients (18% of total number recipients) had implantations in one horn only. Ten recipients (20% of total number of recipients) did not show any embryonic mortality from 8 to 15 days of pregnancy. In four recipients all implantations were degenerating at 15 days p.c.; these represented recipients with one or two implantations.

Rate of embryonic survival was based upon the number of viable embryos (at 15 days p.c.) expressed as a percentage of the number of implantations (at 8 days p.c.).

*Effect of number of implantations at 8 days p.c.* The recipients were grouped into 3 categories according to number of implantations in both uterine horns at 8 days p.c., namely from 1 to 5; from 6 to 10 and from 11 to 16 implantations. Average number of corpora lutea on both ovaries was similar in the 3 groups, ranging from 11.4 to 12.2. Average implantation number in the 3 groups was 3.3, 7.6 and 12.3 respectively (Table I). Average rate of embryonic survival in the 3 groups was 56, 74 and 76%. There was no significant difference between the groups as judged by analysis of variance.

However, the data of the 3 groups were then pooled and correlation and regression were calculated between number of implanta-

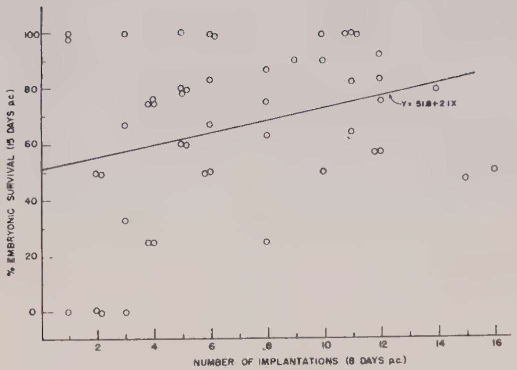

FIG. 1. Regression line between No. of implantations and embryonic survival. Each circle represents one recipient. There was a significant positive correlation.

tions and embryonic survival (Fig. 1). Rate of embryonic survival was positively correlated with number of implantations (r = 0.290, n = 49); this was significant at the 5% level. It was also noted that the location of the non-viable embryos was not related to any specific site along the uterine horn (near the tubal or cervical end) (Fig. 2).

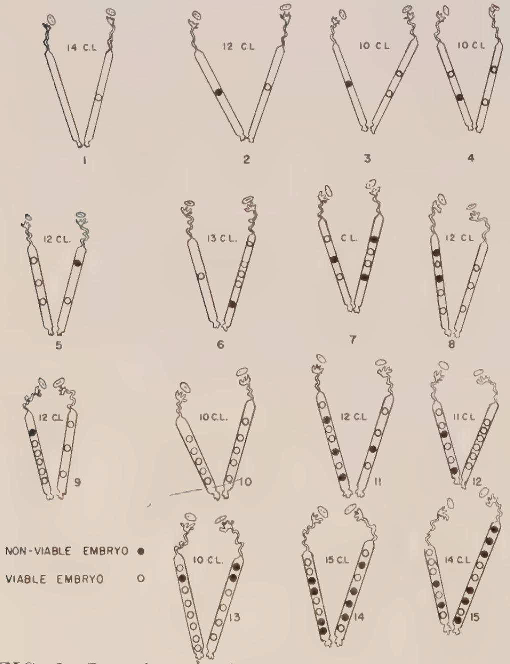

FIG. 2. Location of viable and degenerating embryos at 15 days p.c. in recipients with different numbers of implantations. Note that degeneration of embryos was not related to any specific site in uterine horn.

*Effect of diameter of implantation sites at 8 days p.c.* The diameter of implantation sites is indicated here as the "index of uterine swellings" (I.U.S.). This index, calculated for each litter, represented the average diameter of implantation sites (uterine swellings) which survived from 8 to 15 days p.c. The "index of uterine swellings" for the 3 groups of recipients was 8.8, 9.4 and 9.4 mm respectively (Table II and Fig. 3).

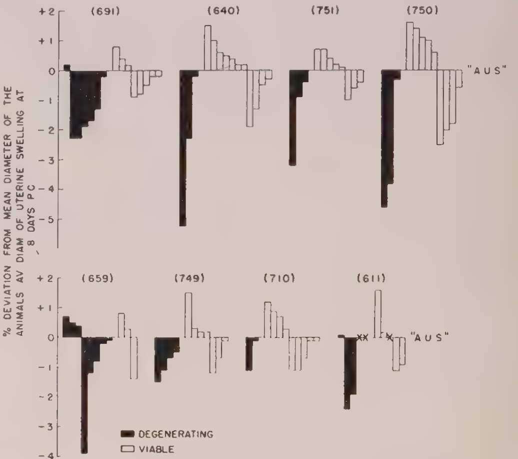

FIG. 3. Diameter of implantation swelling in relation to embryonic survival at 15 days post coitum. The 2 horizontal lines "A.U.S." represent mean diameters of uterine swellings (at 8 days p.c.) which eventually were detected as viable embryos at 15 days p.c.; the lines represent avg for each litter. Each embryo is represented by one column. The columns represent % deviation of swelling diameter (8 days p.c.) from the mean of its litter. Eight litters are represented; each litter included both degenerating and viable embryos. For instance, No. 691 had 1 degenerating embryo with a swelling diameter larger than the mean; and 6 swellings less than the mean; 3 viable embryos with swelling diameter larger than the mean and 5 swellings less than the mean. Most of the degenerating embryos had a smaller diameter of uterine swellings at implantation. × indicates swelling of a diameter to the average.

It appears that, within litters of average size, embryonic survival is not primarily related to number of implantations. There is a "ceiling value" for the number of implantation sites which can be successfully maintained. Beyond a certain level, the mortality tends to fall on the "litter" as a unit rather than on individual embryos, which by competition *inter se* prejudice each other's survival(8). This phenomenon seems more pronounced in excessively large litters in

TABLE II. Embryonic Survival in Relation to Diameter of Swellings at Implantation Site.

| Items | | No. of implantations/recipient | | |
|---|---|---|---|---|
| | | 1–5 | 6–10 | 11–16 |
| Total No. of implantations* | | 75 | 228 | 138 |
| Index of implantation swelling (I.U.S.)† | Range | 5.5–13.0 | 6.6–12.8 | 7.1–12.7 |
| | Mean | 8.8 | 9.4 | 9.4 |
| Uterine swellings which did not survive until 15 days p.c. | | | | |
| % over mean of I.U.S. | | 17 | 23 | 12 |
| % below " " " | | 80 | 75 | 83 |
| % equal to mean | | 3 | 2 | 5 |

* At 8 days post coitum.

† Diameter of swellings of implantations at 8 days p.c. which survived until 15 days p.c.

which the placental development may be adversely affected or the normal vascular supply is inadequate. It is possible that the "ceiling value" for embryonic survival varies with breed, strain and individual as well as it varies with species. Among women, it is well known that some individuals tend to abort repeatedly(9).

In 75 to 83% of the cases, the embryos degenerating at 15 days p.c. had at 8 days p.c., implantation diameters less than the average diameter of uterine swellings at 8 days p.c. This may indicate that most, but not all, the small implantations at 8 days p.c. are susceptible to embryonic death. However, the results do not show whether embryonic death at this stage is a result of retardation in placental development or that degeneration occurs very close to implantation with subsequent retardation of placental development.

Unilaterally ovariectomized females gestate the normal number of live embryos per pregnancy(10): since in such pregnancies, one uterine horn is empty, the average number of embryos gestated in the other horn is doubled. In the mouse, when the number of implantations in one horn is increased artificially above the normal level by ova transfer, embryonic survival is reduced in the other horn so that on an average the total number in the 2 horns combined does not exceed average litter size of the breed(7,11).

Attempts to increase litter size of a small-sized breed of rabbits by increasing the number of ova transferred were unsuccessful and, in fact, produced opposite effects(12). However, in superovulated sheep, with the increasing number of implants, number of surviving embryos reached a ceiling at a level of about 4 per pregnancy(13). This is nearly three times the normal litter size of sheep. The effects of endogenous and exogenous levels of steroid hormones on embryonic survival is still not fully understood.

*Summary.* Variable numbers of fertilized healthy ova (3 to 20) were transferred into 49 recipient rabbits of a synchronous stage of pseudopregnancy. The recipients were laparotomized at 8 days *post coitum* (p.c.) to record number and diameter of implantation sites. At 15 days p.c., the recipients were killed and number of viable embryos recorded. In 3 groups of recipients the number of implantations at 8 days p.c. was 3.3, 7.6 and 12.3 and rate of embryonic survival was 56%, 74% and 76% respectively with no significant difference between the groups. Embryonic survival tended to be positively related to number of implantations. Nonviable implantations were not located at specific sites along the uterine horn. Diameter of implantation swellings was not related to number of implantations per recipient. In 75 to 83% of the cases, the degenerating embryos (15 days p.c.) had implantation diameters (8 days p.c.) less than the average of viable implantation swellings.

Thanks are due to Ayerst Laboratories for donation of pregnant mare serum Equinex, Abbott Laboratories for Vetrophin, Fort Dodge Laboratories for Gonamone, and Upjohn Co. for Gonadogen. Thanks are also due to R. E. Mauer for technical assistance.

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Received May 29, 1961. P.S.E.B.M., 1961, v107.

## Propagation of Junin Virus, the Etiological Agent of Argentinian Hemorrhagic Fever, in HeLa Cell Cultures. (26728)

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During the late fall and early winter of 1958, an epidemic outbreak of a hemorrhagic, febrile illness occurred in the province of Buenos Aires, Argentine Republic. From the blood of several patients, strains of presumably one virus were isolated by Parodi *et al.*(1) and Pirosky *et al.*(2). Studies by these workers showed that the virus, which has been named Junin, was pathogenic for guinea pigs, newborn mice, and chick embryos. Their reported observations, however, as well as work in these laboratories, indicate that serial propagation of Junin virus in these species is not without difficulties. The incubation period in 1-day-old mice is from 10 to 15 or more days; furthermore, numerous animals survive inoculation at various serial dilutions of virus, with resulting irregular titrations.

Other workers(3) have also reported failure of Junin virus multiplication in monkey kidney tissue cultures and KB and HeLa cells. Nevertheless, it was decided to attempt propagation of the virus in HeLa cells according to methods that have been successfully applied in these laboratories to the study of arthropod-borne viruses(4,5).

*Materials and methods. Virus*: The present studies were done with strain *XJ* of Junin virus, supplied by Dr. Parodi. Similar investigations with strains *R5* and *F*, supplied by Dr. Pirosky, have yielded preliminary results closely resembling those obtained with strain *XJ*.

*Immune sera*. Immune sera against Junin virus were produced in mice and rabbits. Mice were given 3 injections of a 10% suspension of infected newborn mouse brain, the first 2 injections intracerebrally at a 3-week interval and the 3rd intraperitoneally; mice were bled 8-15 days after the last injection. Rabbits were inoculated intravenously with 0.5 ml of a 10% suspension of infected mouse brain in physiological saline once a week for 3 consecutive weeks, and exsanguinated 1 month after the last inoculation. Immune sera against other viruses were prepared in the same manner to serve as controls.

*Tissue cultures*. The stable cell line, strain HeLa (Gey), used throughout was obtained from The Tuskegee Institute in 1954 and has been transferred regularly, first at Division of Laboratories and Research, New York State Dept. of Health, Albany, and since 1957 in these laboratories.

The cells were grown in 160-ml French square bottles in a growth-promoting medium consisting of 45% Hanks' balanced salt solution, 15% tryptose phosphate broth (Difco), 30% pooled human adult serum, and 10% fetal bovine serum, supplemented with 100 units of penicillin, 100 μg of streptomycin, and 25 μg of mycostatin per ml. The pH of the Hanks' balanced salt solution was 7.2 to 7.4 and that of the growth-promoting medium about 7.4. The cell inoculum per stock bottle was 10 ml of a cell suspension containing 50,000 cells per ml. The growth-promoting medium was added in amounts of 10 ml per bottle and was changed twice weekly; cellular transfers were carried out regularly at weekly intervals. For cultures of the slant tube type (150 × 16 mm), trypsin-dispersed cells, 200,000 cells per ml, were dispensed into each tube. In carrying out the cell count, an estimate was made of the number of nuclei in occasional cell clumps. After an initial period of 48-72 hours' incubation at 37°C, monolayers of vigorously growing cells were present and ready to be inoculated.

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Inoculation of tubes was carried out as follows: the growth-promoting medium was poured off, leaving behind about 0.2 ml of fluid to which was added 0.7 ml of maintenance medium and 0.1 ml of virus inoculum. The diluent for the latter consisted of 0.75% bovine plasma albumin in phosphate-buffered saline at pH 7.2. The maintenance medium was made up of 97% Eagle's medium(6) and 3% fetal bovine serum supplemented with the same amounts of antibiotics as used in the growth-promoting medium. The pH of the maintenance medium varied between 6.2 and 6.4 and was adjusted with TRIS (Tri(hydroxymethyl)aminomethane), an organic carbon dioxide buffer which has been reported capable of lowering $pCO_2$ *in vivo*(7) as well as *in vitro*(8). TRIS was prepared as a 2 *M* solution in demineralized water, filtered through a Seitz pad, and added in amounts of 0.3 ml to 100 ml of the maintenance medium so as to maintain the pH of inoculated cell cultures at 7.2 ± 0.2 during the period of viral synthesis; however, the amount necessary to obtain a physiologic hydrogen-ion concentration varied somewhat.

The pH of the growth-promoting and maintenance media was measured with a Beckman glass electrode pH meter, and that of inoculated cultures was determined colorimetrically, using phenol red in the medium. Cell cultures were examined at intervals of 2 to 3 days and fluids were changed 3 times a week, occasionally daily. The cell cultures were observed for 21 to 28 days after the beginning of the experiment.

Cell cultures were grown on coverglasses placed in Leighton tubes and treated as described(9) for examination by the fluorescent antibody method(10) and by tinctorial methods.

*Fluorescent antibody technic*[†]: The serum from a rabbit immunized with Junin virus and a horse antirabbit globulin labeled with fluorescein isothiocyanate adsorbed twice with 100 mg of mouse liver powder on the day of use, were employed in these studies. HeLa cell cultures inoculated with the 5th-passage virus were harvested 7 days later, washed, fixed with acetone for 10 minutes at room temperature, and kept at 4°C until treated with the serum; uninoculated cell cultures were similarly prepared. Both inoculated and uninoculated cells were treated simultaneously, and duplicate specimens were used for all dilutions of serum employed.

The acetone-fixed cells on the coverslips were treated for 30 minutes at room temperature with the Junin virus antiserum at dilution 1:2 in phosphate-buffered saline, pH 7.6. The cells were next washed for 10 minutes with several changes of phosphate-buffered saline, pH 7.4, and treated for 30 minutes at room temperature with the labeled globulin at dilutions 1:2, 1:4, and 1:8. The coverslips were washed as before and the excess fluid removed. The coverslips were then mounted on slides in phosphate-buffered glycerol at pH 7 and examined under the fluorescence microscope on the same day.

*Results. Cytopathogenic changes induced by the virus.* Strain *XJ* in the 30th newborn mouse passage was inoculated into cell cul-

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[†] The immunofluorescence microscopy was kindly carried out by Dr. Fred Rapp, Philip D. Wilson Research Foundation, The Hospital for Special Surgery, New York; results of fluorescent antibody and tinctorial studies will be published in detail elsewhere.

TABLE I. Serial Passage of Virus Strain *XJ* in HeLa Cells.

| No. of passage | Cumulative No. of days in tissue culture | TCD$_{50}$ ($-\log_{10}$/ml) | Cumulative $\log_{10}$ of dilution of original inoculum* |
|---|---|---|---|
| 1 | 16 | 5.7 | 5 |
| 3 | 51 | 3.5 | 13 |
| 5 | 71 | 4.5 | 17 |
| 7 | 96 | 4.0 | 21 |
| 9 | 119 | 5.0 | 24 |
| 10 | 132 | 4.5 | 25 |

* Original inoculum was a 10% suspension of infected mouse brain.

tures as $10^{-1}$ to $10^{-5}$ dilutions of infected mouse brain. Within 5 to 15 days, characteristic changes were observed in the cell cultures. A 1:100 dilution produced cytopathogenic changes by the 5th day in each of the 3 cultures inoculated. Examination of the unstained cell cultures under low magnification (×125) revealed small discrete focal areas of shrunken and granulated cells within the sheet-like outgrowth of cells (Fig. 1, 2). Later, the affected cells tended to fall off the tube wall, with gradual formation of macroscopically recognizable holes. At the end of the 3rd week the whole monolayer was involved, only small islands of normal cells remaining.

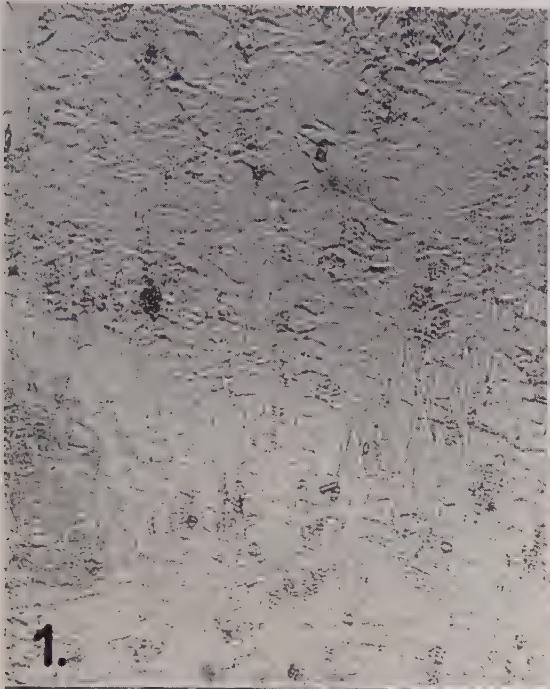
The *XJ* strain did not require adaptation to HeLa cells, and over a 4-month period 10 passages have been made in cell cultures with the use of 0.1 ml of either diluted fluid phase or combined cell and fluid phase. This has resulted in dilution of the original inoculum to $10^{-25}$ (Table I), a value far exceeding the LD$_{50}$ of the source newborn mouse brain suspension. Transfers from infected to new cultures were carried out at 7- to 14-day intervals. Estimation of the virus at the 5th-passage level gave a TCD$_{50}$ titer of $10^{-4.5}$ per ml of combined fluid and cell phase. It appears that slightly higher TCD$_{50}$ titers can be obtained by combining the cells and the fluid phase rather than titrating the fluid phase alone.

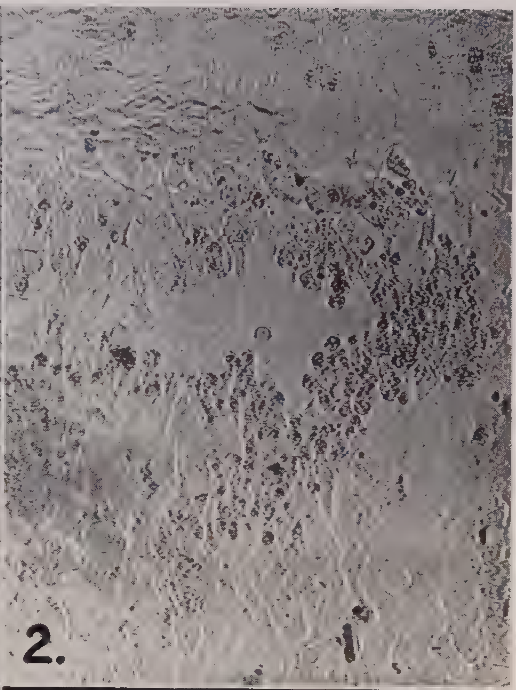
Examination of coverglass preparations inoculated with 5th-passage material and stained with Giemsa revealed significant cytoplasmic changes in cells localized within the focal lesions. In such cells, polymorphic basophilic bodies of varying size occurred either near the nuclear membrane or irregularly throughout the cytoplasm (Fig. 3).

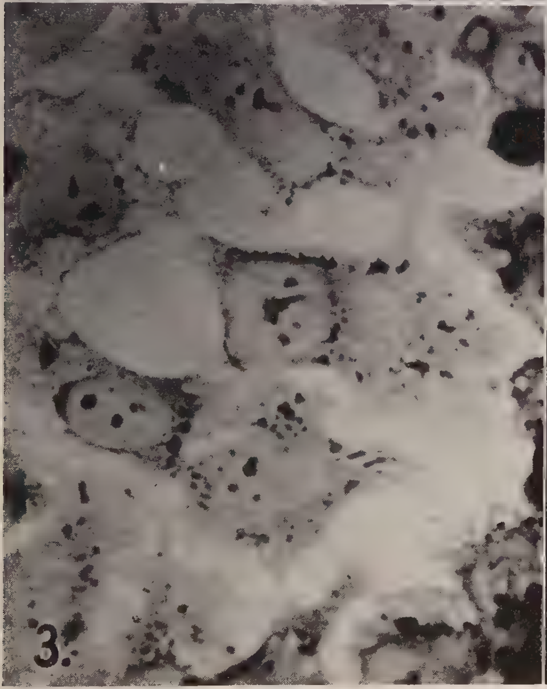
In fluorescent antibody studies, uninoculated cultures exhibited a diffuse green background, but observations at higher magnification (×400) failed to reveal any localization of antigen in individual cells. In sharp contrast, specific localization of antigen occurred in the infected cultures treated with globulin at dilutions 1:2 and 1:4. Staining was very weak when the labeled reagent was diluted 1:8. Specific staining consisted of antigen aggregates of various sizes in the cytoplasm (Fig. 4). Intranuclear localization of antigen was not evident, and nucleoli showed up very clearly as pale white bodies. The cytoplasmic granules varied considerably in size, from very small bodies to rather large, brightly-staining units (Fig. 4). In large areas of the sheet, the cells did not show specific cytoplasmic fluorescence; cells with fluorescent staining occurred mainly in foci, occasionally as single cells.

*Neutralization of the agent propagated in cell cultures by specific immune sera.* Additional evidence that the agent propagated *in vitro* was Junin virus, was obtained from neutralization tests. Neutralization of cytopathogenicity has been observed with both mouse and rabbit immune sera. In one test, immune and control rabbit sera were inactivated at 56°C for 30 minutes. Equal amounts of increasing 10-fold dilutions of virus (10% suspension of 30th passage newborn mouse brain) and undiluted serum were mixed and incubated at 37°C for 1 hour. One-tenth ml of the serum-virus mixtures was inoculated into each of 3 HeLa cell cultures per virus dilution. Cell cultures were examined daily and discarded 21 days after the beginning of the test. Results showed that the undiluted serum from a rabbit immunized with Junin virus neutralized 3 log units of the cytopathogenic agent, which reached a titer of 2.5 TCD$_{50}$ ($-\log_{10}$ per ml). The titer in the control tubes with normal rabbit serum was 5.5.

In an additional experiment, virus from the 8th passage in HeLa cells was used. Immune and control rabbit sera were inacti-







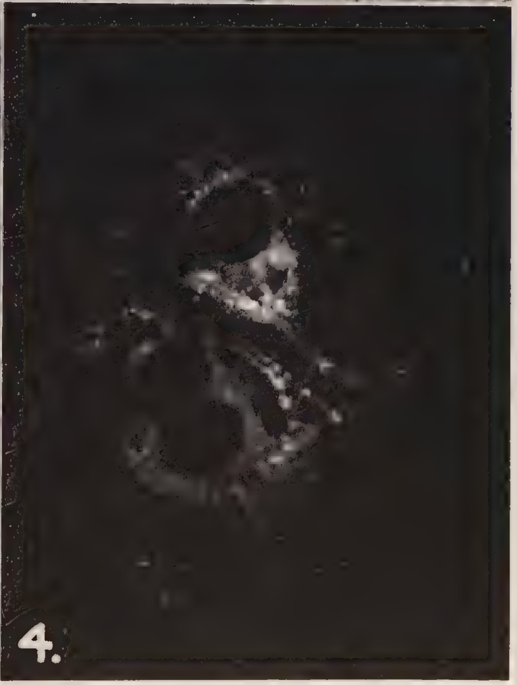


FIG. 1. Uninoculated HeLa cells (×108) at 11 days.

FIG. 2. HeLa cells (×108) inoculated with 2nd passage strain *XJ* virus. Area of focal lesion 11 days after inoculation.

FIG. 3. HeLa cells (×1080), Giemsa stained, 7th day after inoculation with 6th passage strain *XJ* virus. Note basophilic cytoplasmic inclusions.

FIG. 4. Fluorescent antibody preparation of HeLa cells (×1080), 7th day after inoculation with 6th passage strain *XJ* virus. Note specific localization of antigen in cytoplasm.

vated as before. Equal amounts of a 1:10 dilution of combined cell and fluid phase were mixed with increasing 2-fold dilutions of serum, incubated for 1 hour at 37°C, and inoculated into cell cultures. Within 15 days, a 1:40 dilution of the serum from a rabbit immunized with Junin virus neutralized 30 $TCD_{50}$ doses as determined by parallel titration of the virus.

It has been observed that the $TCD_{50}$ titers of Junin virus propagated in 1-day-old mice and titrated in HeLa cells exceed by an average of 1 to 2 log units the $TCD_{50}$ titers of the virus serially maintained in cell cultures and similarly titrated. Under the circumstances, it would seem advisable to use infected mouse brain as source of virus for neutralization tests in which increasing virus dilutions are employed, but to use virus from HeLa cell cultures in the constant virus—varying serum technic and for fluorescent antibody studies.

*Effect of sodium desoxycholate on Junin virus.* Prior to this study, attempts to determine whether Junin virus was inactivated by sodium desoxycholate had been inconclusive, owing to the irregular outcome of inoculation of serial dilutions of virus in mice. Since sensitivity to this chemical is a property of arthropod-borne viruses(11), it was decided to make use of HeLa cell cultures to obtain definite evidence.

To 1 part of a 10% suspension of mouse brain infected with Junin virus was added 1 part of a 1% solution of sodium desoxycholate. Two control mixtures were prepared, with virus and diluent instead of sodium desoxycholate and with the chemical plus diluent instead of virus. After incubation for 1 hr at 37°C, all mixtures were diluted by increasing 10-fold dilutions to $10^{-7}$; 0.1 ml of each dilution was inoculated into each of 2 cell cultures which were examined daily for 15 days. In a similar experiment, virus from the 2nd passage in HeLa cells was used. In the absence of sodium desoxycholate, the virus propagated in mice and in cell cultures had titers ($TCD_{50}$ per ml) of $10^{-4.5}$ and $10^{-4.0}$, respectively; in the presence of sodium desoxycholate, the same virus lines failed to multiply. Rounding and granulation of cells was present in the tubes inoculated with the undiluted mixtures; that this was due to a toxic action of the chemical on the cells and not to virus multiplication was indicated by the fact that control cell cultures inoculated with 1% sodium desoxycholate showed identical changes.

*Summary.* Junin virus, the etiological agent of Argentinian hemorrhagic fever has been propagated serially in HeLa cell cultures. Appropriate cell viability during the long experimental period was obtained by the use of a suitable combination of growth-promoting and maintenance media as well as by frequent fluid changes during the period of viral synthesis.

A characteristic cytopathic effect was observed during the 1st passage and did not change during 10 consecutive transfers of the agent. Evidence that the cytopathogenic agent was Junin virus was derived from fluorescent antibody studies and from neutralization tests with sera from animals immunized with the virus propagated in newborn mice. In cell cultures, Junin virus was inactivated by sodium desoxycholate, a property common to all arthropod-borne viruses.

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Received April 20, 1961. P.S.E.B.M., 1961, v107.

# Effect of Hypophysectomy and Growth Hormone on Glucose Uptake by Rat Epididymal Fat Tissue.* (26729)

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(Introduced by Richard C. de Bodo)
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The effect of hypophysectomy and of growth hormone on carbohydrate utilization has been the subject of numerous studies, both *in vivo* and *in vitro*. A critical analysis of such investigations appeared recently (1,2). The *in vitro* studies, performed mainly on the cut rat diaphragm, gave conflicting results in that some workers reported that diaphragms from hypophysectomized rats took up more glucose than normal diaphragms(3-5), while others found a diminished(5) or normal(6) uptake. More recently, it has been reported that the uncut "intact" diaphragms(7) or perfused hearts (8) of hypophysectomized rats took up less glucose than the respective tissues of normal rats.

The present studies have extended these investigations to rat epididymal fat tissue. This tissue is very active metabolically and is sensitive to insulin(9). The glucose uptake by epididymal fat pads excised from normal, hypophysectomized and growth hormone-treated hypophysectomized rats was determined. In addition, the effect of serum from these animals on glucose uptake was determined in an attempt to correlate it with the changes in glucose uptake by the excised tissues.

*Materials and methods.* Epididymal fat tissue was obtained from normal and hypophysectomized male Sprague-Dawley rats (purchased from Hormone Assay Laboratories, Inc.), weighing 150-225 g and fed *ad lib.* The hypophysectomized animals were used 7-21 days following operation. Animals receiving growth hormone were injected with 30 μg of bovine growth hormone, i.p., (Somar A, Lot R50109; gift of Endocrinology Study Section, Nat. Inst. of Health) 24 hr prior to sacrifice. The animals were decapitated and their blood was collected and allowed to clot. Serum was removed, pooled, and frozen for later use. Each epididymal fat pad was removed quickly, cut into 2 or 3 sections weighing 50-100 mg, and put into preweighted vials containing 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with a glucose concentration of 100 mg %. Vials were immediately reweighed to determine tissue weight. They were then incubated for 2 hr at 37°C with constant shaking. A control vial, containing the incubation medium, but no fat tissue, was also incubated. At the end of incubation, the vials were placed in an ice bath to terminate further glucose uptake. The glucose concentration of each sample was determined in aliquots of Somogyi zinc-barium filtrate(10), by the method of Nelson(11). Glucose uptake is expressed as μg glucose disappearing/g tissue/hr incubation time. The effect of serum on glucose uptake was measured by incubating epididymal fat tissue from normal rats with the test serum and comparing glucose uptake from the serum with glucose taken up from the glucose-buffer solution. Each serum sample was assayed against a control glucose-buffer sample using fat tissue from the same animal.

*Results.* The effect of hypophysectomy and growth hormone treatment on glucose uptake by rat epididymal adipose tissue is shown in Fig. 1. Glucose uptake by adipose tissue from the hypophysectomized animals was 733 ± 39 μg/g/hr. This is a significant ($P = <0.01$) decrease from the glucose uptake by normal tissue which is 1165 ± 88 μg/g/hr. A single injection of growth hormone (30 μg) in hypophysectomized animals, 24 hr prior to sacrifice, increased significantly ($P = <0.01$) the glucose uptake by the excised fat pads to 970 ± 60 μg/g/hr.

The effect of serum from hypophysectomized and growth hormone-treated hypophysectomized rats on glucose uptake by normal

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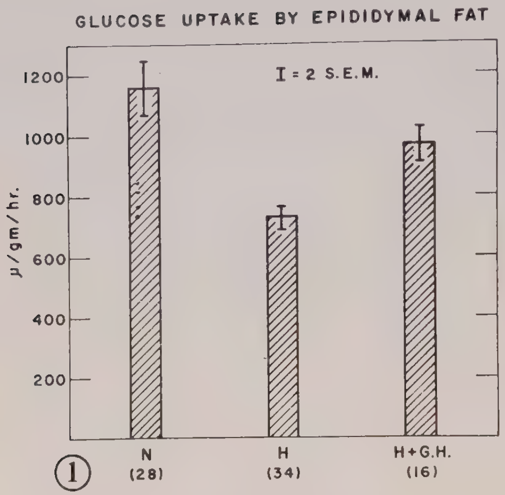
* This investigation was supported by grant from U.S.P.H.S.

† Senior Research Fellow, U.S.P.H.S.



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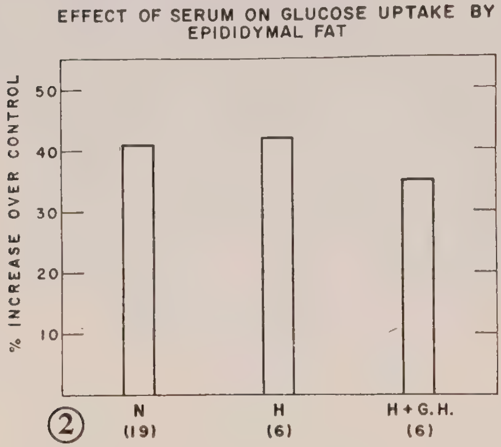

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FIG. 1. Glucose uptake by epididymal fat tissue of normal (N), hypophysectomized (H), and growth hormone-treated hypophysectomized (H + G.H.) rats. Uptake is expressed as μg glucose disappearing/g adipose tissue/hr incubation. Numbers in parentheses represent No. of experiments. S.E.M. = Stand. error of the mean.

FIG. 2. Effect of sera from normal (NS), hypophysectomized (HS), and growth hormone-treated hypophysectomized (HS + G.H.) rats on glucose uptake by normal epididymal fat tissues. Activity is expressed as % increase in glucose uptake above that observed in glucose-buffer medium. Numbers in parentheses represent No. of experiments.

fat tissue is shown in Fig. 2. Values are expressed as % increase in glucose uptake over that obtained with normal fat pads incubated in glucose-buffer media. Glucose uptake in the latter was 1025 ± 94 μg/g/hr. It is evident that the sera from normal animals increased glucose uptake by 41% above control level. The sera from hypophysectomized rats, fed *ad lib.*, increased glucose uptake to the same extent as those from normal rats. Likewise, the single injection of growth hormone did not influence the effect of sera on glucose uptake.

*Discussion.* The observed diminished glucose uptake by the fat tissue of the hypophysectomized rat agrees with the recent findings that the excised "intact" diaphragm(7) and the perfused heart(8) of the hypophysectomized rat take up less glucose than the respective tissues of normal rats. It is also in harmony with the *in vivo* findings of Steele *et al.*(12) that in the hypophysectomized dog in the steady postabsorptive state, rate of endogenous glucose removal from the plasma is lower than in the normal dog.

The decreased uptake of glucose by the tissues of the hypophysectomized animal cannot be ascribed at present to a deficiency of any one specific hormone. The characteristic atrophy of the thyroid glands found in these animals, results in an over-all decrease in metabolism, which may contribute to the observed decrease in glucose uptake by the excised adipose tissue. It is also probable that the decreased uptake of glucose reflects a compensatory decrease in insulin secretion. Supporting this possibility is the demonstration of atrophy(13) and decrease in size of the Golgi bodies(14) of the pancreatic beta cells of hypophysectomized rats.

The present finding that administration of growth hormone to the hypophysectomized rat increases the low glucose uptake of the excised epididymal fat tissue is in agreement with the *in vivo* observation by Altszuler *et* al.(15,16) that in the hypophysectomized dog a growth hormone regimen increases rate of uptake of plasma glucose by the tissues, concomitant with an increase in hepatic glucose production. The increased uptake of glucose by the tissue of these growth hormone-treated animals may be due to increased insulin secretion. There is substantial evidence that growth hormone stimulates secretion of insulin (for references see 17). However, growth hormone administration was shown also to lower blood glucose concentration in acutely depancreatized dogs (18) and hypophysectomized-eviscerated rats(19). Also, addition of growth hormone *in vitro* increases glucose uptake by the diaphragm from normal(20) and hypophysectomized(19) rats and by epididymal fat tissue from normal rats(21).

The present studies reveal that sera from hypophysectomized rats increase glucose uptake by normal epididymal tissue to the same extent as sera from normal rats. Since the ability of serum to increase glucose uptake by fat tissue has been utilized as an index of "insulin-like" activity(22) it would appear that sera of hypophysectomized animals have the same activity as sera from normal animals. This finding is at variance with that of Randle(23), who reported that in a majority of his hypophysectomized rats no insulin activity could be detected in the plasma. The absence of insulin activity is not readily explainable, since his rats were not fasted and insulin was presumably being secreted. In view of the lack of specificity of the aforementioned method of insulin assay, it is feasible that extirpation of the pituitary gland may remove or reduce the effectiveness of contra-insulin agents, thereby giving the appearance of a normal insulin-like activity, even though the actual amount of insulin in the serum may be reduced.

The lack of change in the effectiveness of serum to increase glucose uptake observed at 24 hr following injection of growth hormone agrees with findings of Randle(23). This does not necessarily dispute the possibility that growth hormone increased secretion of insulin, and that this was responsible for the increased uptake by excised fat tissue.. Since 90% of the injected insulin is cleared from the serum within 20 minutes(24,25) and is no longer detectable within 60 minutes(25), it is conceivable that any insulin released as a result of the growth hormone injection may have been cleared from the serum in the 24-hr period following the injection.

*Summary*. The epididymal fat tissue from hypophysectomized rats took up a significantly smaller amount of glucose than did the normal tissue. A single injection of bovine growth hormone into hypophysectomized rats increased glucose uptake by epidymal fat tissue excised 24 hr later. Sera from hypophysectomized and growth hormone-treated hypophysectomized rats increased glucose uptake by normal fat tissue to the same extent as sera from normal animals.

The authors gratefully acknowledge the helpful contributions made by Dr. Richard C. de Bodo.

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Received May 22, 1961. P.S.E.B.M., 1961, v107.

## Relation of Sialic Acid to Rh$_0$ (D) Antigen. (26730)

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Dodd *et al.*(1) reported specific inhibition of Rh$_0$ (D) antibody by sialic acids. We proposed to examine this inhibition using various neuraminic acid derivatives with Rh$_0$ (D) saline antibodies and to investigate the effect of neuraminidase, an enzyme which will remove sialic acid from erythrocytes, on agglutination of the cells by specific antiserum.

*Materials and methods. N-acetylneuraminic acid (NANA)* was prepared from human blood plasma according to Martensson *et al.*(2). Crystallization from glacial acetic acid yielded crystals with a melting point of 184-185°C. No glycolic acid could be detected by the method of Klenk and Uhlenbruck(3). The material moved as a single spot when analyzed by paper chromatography in the following solvents: butanol: acetic acid:water (4:1:5), Rf 0.32; butanol: propanol:0.1 N HCl (1:2:1), Rf 0.45. *Crude bovine sialic acid* (I) was prepared from mucin of bovine submaxillary glands according to Blix *et al.*(4). The dried mucin was suspended in water, sialic acid was liberated by acid hydrolysis and isolated by ion exchange chromatography(5). This material assayed 74% NANA by the resorcinol reaction(6). When examined by paper chromatography, 3 components reacting as sialic acid were observed. A small amount of ninhydrin-positive material was also present. The fastest-moving sialic acid component was identified as N,O-diacetylneuraminic acid by the fact that on further acid treatment it was converted to NANA and also gave a hydroxamic acid test for esters. According to glycolic acid analysis, the material contained 14% N-glycolylneuraminic acid (NGNA). *Crystalline bovine sialic acid* was prepared from crude bovine sialic acid (I) by crystallizing it twice from methanol-ether and once from glacial acetic acid. This material had a melting point of 186-187°C. According to glycolic acid analysis the material contained 16% NGNA. Two distinct spots were observed when the crystals were analyzed by paper chromatography in butanol: propanol:0.1 N HCl. The two spots were identified as NANA, Rf 0.45, and NGNA, Rf 0.34. *Crude bovine sialic acid*(II) was prepared by dissolving bovine submaxillary mucin in water and heating at 100°C for 30 minutes. The liberated sialic acid was isolated by methanol extraction(4). This material contained 61% NANA and 9% NGNA. It showed 5 components reacting as sialic acid when analyzed by paper chromatography. Three components were identified as NANA, NGNA, and N,O-diacetylneuraminic acid. The other 2 components are reported by Blix(7) to be triacetylneuraminic acid and an isomerization product. *Porcine sialic acid* was isolated from porcine submaxillary mucin according to Blix(6). The material contained 63% NANA and 45% NGNA. It showed NANA and NGNA spots by paper chromatography. *Human brain gangliosides* were isolated from acetone powders of fresh human grey matter by extraction with methanol-chloroform (2:1), solvent distribution according to Folch *et al.*(8), dialysis and finally silicic acid chromatography. The final product contained 23% NANA, 31% hexose (as galactose), 5.5% hexosa-

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* This investigation was supported by Nat. Inst. Health Grant and by Division of Mental Hygiene and Correction, State of Ohio.

TABLE I. Inhibition of Agglutination of Rh$_0$ (D) Trypsinized Cells by Complete and Incomplete Antibody.

| Inhibitor | Antibody dilution (reciprocal) | | Agglutination | | Inhibitor conc., μg/0.3 ml |
|---|---|---|---|---|---|
| | Incomplete | Complete | Without inhibitor | With inhibitor | |
| NANA | 16 | | 2+ | 2+ | 1012 |
| | 32 | | 1+ | 1+ | 1012 |
| | | 8 | 2+ | 2+ | 1012 |
| | | 16 | 1+ | 1+ | 1012 |
| NANA | 16 | | 4+ | 4+ | 990 |
| | 64 | | 2+ | 2+ | 990 |
| Crude bovine sialic acid (I) | 16 | | 4+ | 3+ | 1000 |
| | 64 | | 2+ | 1+ | 500 |
| Bovine sialic acid (crystalline) | | 32 | 3+ | 3+ | 830 |
| | | 64 | 2+ | 2+ | 830 |
| Crude bovine sialic acid (II) | 16 | | 4+ | 4+ | 996 |
| | 64 | | 2+ | 1+ | 31 |
| | 64 | | 2+ | — | 62 |
| Porcine sialic acid | | 32 | 3+ | 3+ | 986 |
| | | 64 | 2+ | 2+ | 986 |
| Human brain ganglioside | | 16 | 4+/S | 4+/S | 580 |
| | | 64 | 2+ | 1+ | 580 |

mine, and 16% sphingosine. *Antisera*—Rh$_0$ (D) albumin antiserum (incomplete antibody) was obtained from Dade Laboratories, Miami, Fla. Rh$_0$ (D) saline antiserum (complete antibody) was obtained from Knickerbocker Laboratories, New York. Anti-A serum was obtained from Ortho Pharmaceutical Co., Raritan, N. J. *Pneumococcal neuraminidase,* a highly purified preparation, was kindly supplied by Dr. E. H. Eylar (9). *Inhibition and agglutination tests* were performed as described by Bigley *et al.*(10). The materials to be tested were dissolved in phosphate buffered saline and the pH adjusted to 7.2 ± 0.2.

*Results.* As shown in Table I, NANA did not exhibit inhibition in any of the Rh systems tested. Crystalline bovine sialic acid failed to inhibit the agglutination of trypsinized cells with saline antibodies. However, when crystalline bovine sialic acid which had not been neutralized was tested in the same system, lysis occurred in the tube containing the most sialic acid and apparent complete inhibition was observed at a concentration of 500 μg/0.3 ml. Porcine sialic acid also failed to inhibit agglutination.

Slight inhibition was observed with crude bovine sialic acid (I), and with the human ganglioside preparation as shown in Table I. The inhibition observed with crude bovine sialic acid (II), using a 1:64 antibody dilution, could not be demonstrated in a 4+ system with a concentration of approximately 1000 μg/0.3 ml. Furthermore, the inhibition obtained in the 2+ system was erratic; the tube containing 1000 μg of material showed no inhibition. Similar results were obtained in repeated testing.

A sample of Type A, Rh positive cells was washed with saline and suspended in Tris buffer, pH 5.4, containing the neuraminidase. A control sample of cells was suspended in the buffer without enzyme and treated identically throughout the procedure. The cells were incubated at 37°C and at 30, 60, and 120 minutes, aliquots removed, cells centrifuged and the supernatant analyzed for liberated sialic acid with the thiobarbituric acid assay(11). A maximum amount of sialic acid had been liberated after incubation for 60 minutes. A packed cell volume and cell count made it possible to calculate that approximately $4 \times 10^7$ molecules of sialic acid were liberated per cell. The cells were then washed with saline and a 2% suspension in buffered saline was prepared for the agglutination test. The neuraminidase-treated cells were agglutinated more readily than the non-treated cells (Table II). The treated

TABLE II. Effect of Neuraminidase on Agglutination of $Rh_o$ (D) Cells with $Rh_o$ (D) Saline Antiserum.

| Antiserum dilution (reciprocal) | 4 | 16 | 32 |
|---|---|---|---|
| Treated cells | 4+ | 3+ | 2+ |
| Nontreated cells | 2+ | 1+ | — |

and control cells could not be distinguished when tested with anti-A serum. No inhibition of a maximal (4+) or partial (2+) agglutinating dilution with these cells was observed with NANA at a concentration of 1000 μg/0.3 ml.

*Discussion.* The specific inhibition of an antigen-antibody system by a compound is generally accepted as evidence of the complementary fit of the antibody and the inhibitor. No inhibition was observed with either of the crystalline sialic acid preparations or with the porcine sialic acid in the systems tested. It should be noted that slight inhibition was observed only with the impure preparations. Due to the heterogeneity of these materials, no conclusion can be made concerning the substance responsible for the slight inhibition observed.

If sialic acid constitutes a portion of the immunological determinate group of $Rh_o$ (D) antigen, the removal of sialic acid from $Rh_o$ (D) positive cells would be expected to decrease their affinity for $Rh_o$ (D) antibody. Pneumococcal neuraminidase was shown to liberate a large amount of sialic acid from the cells. The enzyme-treated erythrocytes were agglutinated more readily by $Rh_o$ (D) saline antibodies than the non-treated control cells. This phenomenon is similar to the effect produced by trypsin treatment. That trypsin and neuraminidase should affect the cells similarly with respect to Rh agglutination is particularly reasonable in view of recent reports(12,13,14) that a sialic acid-containing peptide is liberated from red blood cells by treatment with trypsin.

The behavior of the neuraminidase-treated erythrocytes and the failure of crystalline sialic acid preparations to inhibit anti-$Rh_o$ (D) agglutination does not support the supposition that sialic acid resembles the immunological determinate group of $Rh_o$ (D) antigen.

*Summary.* Several crude preparations of sialic acid containing different derivatives of neuraminic acid, and 2 crystalline preparations, N-acetylneuraminic acid and bovine sialic acid (containing NANA and NGNA) were tested for their ability to inhibit agglutination of $Rh_o$ (D) red blood cells by $Rh_o$ (D) antibody. Two of the crude preparations showed slight inhibition, while no effect was observed with the crystalline preparations. $Rh_o$ (D) positive erythrocytes were treated with neuraminidase, and sialic acid was shown to be liberated. The neuraminidase-treated cells were more readily agglutinated by $Rh_o$ (D) antibody than nontreated control cells.

We gratefully acknowledge the advice and cooperation of Drs. M. C. Dodd and N. J. Bigley in these studies.

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Received April 26, 1961. P.S.E.B.M., 1961, v107.

# Complement Components of Paired Mother-Cord Sera.* (26731)

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It is well established that the human is particularly susceptible to a variety of infectious agents during the newborn period. The diminished complement activity of cord serum, compared with that in the maternal circulation(1,2,3,4), may emphasize the importance of this substance as a nonspecific humoral factor in resistance(5).

The present work represents an attempt to define the factors which may play a role in the reduced total hemolytic complement activity of cord serum. A study was made of the major complement components (C′1, C′2, C′3, C′4) present in mother and cord sera The latter were also tested for the possible presence of anticomplementary substances.

*Materials and methods.* Blood samples were obtained from 16 pairs of mother and offspring at time of delivery. All were normal, full-term, single birth pregnancies. Birth weights ranged from 2,450-3,820 grams; no cases of fetal distress or perinatal complication were observed. Mothers' ages ranged from 16-36 years; anesthesia, when used, was local. Two samples of blood from the umbilical cord and one from a maternal vein were collected and allowed to clot at room temperature for one hour. The maternal and one cord (Ca) specimens were refrigerated 3-4 hours; the second cord specimen (Cb) for 8-23 hours. The sera were separated, frozen and stored at –20°C until tested. Classical "R" reagents for titrating complement components were prepared from pooled fresh human sera by the method described by Wedgwood(6). All reagents were prepared at the same time, frozen and stored at –20°C in aliquots suitable for individual titrations. Complement activity of each aliquot was determined on the day of component titration with no significant variation in the titers of the reagents noted. In attempting to separate complement components of guinea pig serum by continuous flow paper electrophoresis, it was found that none of the separated fractions would lyse sensitized sheep erythrocytes. Hemolytic activity was restored to those fractions in the $\alpha$-$\beta$ region following addition of heated serum, R3, but not R4. Therefore, C′4 appeared to be the missing factor. These findings were independent of buffer (veronal, phosphate, borate), pH (7.4-8.6), ionic strength (0.02-0.07). An ionic strength of 0.05 was found to be less deleterious to complement than 0.02. We could not elute C′4 from the curtain. Further testing of the fractions with R1 and R2 indicated that C′1, C′2, and C′3 migrated as a peak in the $\alpha$-$\beta$ globulin region. An R4 reagent prepared in this manner appeared to be more specific than the classical ammonia-treated material, for C′4 was completely undetectable within the peak of the other 3 components. The same phenomenon occurred with human serum, but recovery because of dilution was low. Therefore, the last 4 paired sera were tested with the electrophoretically prepared guinea pig R4. Hemolytic complement titers were expressed as the reciprocal of the dilution required to lyse 50% of a standardized sheep erythrocyte suspension in a reaction volume of 5.0 ml. This technic has been described by Taliaferro and Taliaferro(7) for titration of hemolysin. All serum constituents were placed in an ice bath until admixture was complete, sensitized cells added, the tubes centrifuged following incubation at 37°C for one hour, and the hemoglobin concentration of the supernatant portion measured by a Klett-Summerson colorimeter. The 50% endpoint was determined by the method of Taliaferro and Taliaferro (7). Total protein, based on a modification of the Folin-Ciocalteu method(8,9), and electrophoretic patterns (Spinco Model R and Analytrol) were determined when sufficient serum was available. Concentration of the various serum fractions was calculated by division of the analytrol graph first at the

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* This investigation was supported in part by research grant from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

TABLE I. Comparison of Total and Component Activities of Complement Obtained from Maternal and Corresponding Cord Sera.

| Source* | Time between collection and removal of serum (hr) | Time stored at –20°C (days) | Hemolytic complement (C'H50 units/ml) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Total | C'1 | C'2 | C'3 | C'4 |
| M 1 | 4 | | 120 | 315 | 144 | 190 | |
| Ca | 4 | 35 | 57 | 120 | 60 | 79 | |
| Cb | 8 | | 52 | 112 | 55 | 100 | |
| M 2 | 4 | | 102 | 345 | 87 | 144 | |
| Ca | 5 | 35 | 40 | | | | |
| Cb | 23 | | 31 | | | | |
| M 3 | 5 | | 59 | 120 | 32 | 83 | >900 |
| Ca | 5 | 36 | 37 | 79 | <19 | 79 | >600 |
| Cb | 19 | | 32 | 79 | <19 | 63 | >600 |
| M 4 | 5 | | 63 | 123 | 44 | 114 | >900 |
| Ca | 5 | 36 | 32 | 74 | <19 | 55 | >600 |
| Cb | 19 | | 25 | 66 | 30 | 46 | >600 |
| M 5 | 4 | | 63 | 114 | 72 | 114 | >800 |
| Ca | 4 | 42 | 29 | 50 | 10 | 50 | >900 |
| Cb | 18 | | 21 | | | | |
| M 6 | 4 | | 76 | 181 | 60 | 125 | >800 |
| Ca | 5 | 40 | 35 | 63 | <15 | 63 | >900 |
| Cb | 9 | | 33 | | | | |
| M 7 | 4 | 35 | 63 | 144 | 17 | 100 | >1600 |
| Ca | 4 | | 25 | 44 | 16 | 42 | > 900 |
| M 8 | 4 | | 100 | 44 | 104 | 144 | >3200 |
| Ca | 4 | 57 | 42 | 250 | 87 | 79 | >2400 |
| Cb | 7 | | 45 | | | | |
| M 9 | 4 | | 65 | 122 | 109 | 131 | >3200 |
| Ca | 4 | 57 | 34 | 173 | 56 | 44 | >2400 |
| Cb | 20 | | 22 | | | | |
| M 10 | 4 | | 72 | 131 | 79 | 125 | >3200 |
| Ca | 4 | 50 | 58 | 330 | 83 | 89 | >2400 |
| Cb | 22 | | 44 | | | | |
| M 11 | 4 | | 87 | 177 | 117 | 198 | >3200 |
| Ca | 4 | 55 | 63 | 125 | 131 | 134 | >2400 |
| Cb | 22 | | 57 | | | | |
| M 12 | 4 | | 89 | 104 | 138 | 100 | >3200 |
| Ca | 4 | 47 | 38 | 83 | 81 | 87 | >2400 |
| Cb | 22 | | 32 | | | | |
| M 13 | 5 | 7 | 109 | | | | 1500† |
| C 13 | 5 | | 56 | | | | 650† |
| M 14 | 5 | 6 | 93 | | | | 870† |
| C 14 | 5 | | 50 | | | | 480† |
| M 15 | 5 | 2 | 109 | | | | 1145† |
| C 15 | 5 | | 60 | | | | 575† |
| M 16 | 5 | 2 | 131 | | | | 1470† |
| C 16 | 5 | | 63 | | | | 790† |

* M, maternal serum; C, cord serum; Ca and Cb differ only in time of serum removal. Numbers indicate sets.

† Tested with electrophoretically prepared guinea pig reagent.

origin of the gamma-globulin curve, next at the base of the albumin peak, with the distance between these reference points again equally divided. Thus an arbitrary but relatively standard separation of the albumin and alpha-, beta-, and gamma-globulins was achieved. Possible anticomplementary activity of cord sera was determined in 2 ways: by delaying the removal of serum from one sample (Cb) and allowing it to stand for a longer period than Ca(1); secondly, in 3 cases, by addition of cord aliquots to both homologous and heterologous maternal sera and testing the resultant mixture for total hemolytic activity.

*Results.* Total complement and component concentrations of each maternal serum were compared only to that of the corresponding umbilical cord sample. The results are shown in Table I. Maternal titers ranged from 1.2-2.6 times that of their infants with an average 2-fold difference (79.9 *vs* 40.8 units).

The titer of the first component (C′1) in 9 mothers varied from 1.2-3.3 times that of their infants; in 3 cases, tested on the same day, cord sera content was greater than maternal. Maternal C′2 activity ranged from 0.9 to approximately 7 times that of the infant. The third component (C′3) of maternal serum was, without exception, more active (1.1-3.0) than that of cord sera.

In testing the first 12 pairs of sera for the fourth component (C′4), a 50% endpoint was not determined; the highest dilution used in each case produced more than 50% hemolysis. For this reason, the 100% hemolysis endpoints are given (Table I). It was observed that total lysis occurred in the maternal samples to a higher dilution than with the cord. The last 4 pairs were tested with the guinea pig reagent. The maternal 50% hemolysis titers were higher (1.8-2.3) than the infants. The titers with human R4 were greater than those found with the guinea pig reagent. This could be due to incomplete compatability with the latter material, although Ecker(10) states that apparently all components of human and guinea pig sera are mutually interchangeable. However, the ratios within each pair of sera were reproducible and probably represent valid differences.

Results of the tests for anticomplementary activity of cord sera are shown in Table I. The Cb specimen of each set, allowed to stand for a longer period of time between collection and separation of the serum, appeared to lose complement activity in almost every case. In the one exception, the titers were approximately the same. This is in contrast to the observations of Nattan-Larrier *et al.*(1) who proposed a decrease in an anticomplementary substance with the resultant increase in titer if these procedures were followed. Furthermore, it appeared (Table II) that mixing equal volumes of cord and maternal sera resulted in a complement titer only slightly less than that expected. This would indicate that anticomplementary activity of fetal serum is not a major factor in the lower titers observed in these sera relative to the corresponding maternal sera.

Protein concentration and electrophoretic distribution of the various fractions was determined on 7 pairs of sera. The results are shown in Table III. Total protein varied from 6.6-9.0 g % in maternal serum, and from 6.4-7.9 g % in the umbilical cord specimens. No constant maternal/cord ratio was apparent. In general, the concentration of albumin was greater in cord serum, whereas the reverse situation occurred with the alpha- and beta-globulins. The concentration of gamma-globulin was variable with inconstant differences between the pairs of sera.

*Discussion.* The lower total hemolytic complement activity of cord serum is thought by some(4) to be a reflection of incomplete equilibration by placental transfer. Others (1) suggest the level is influenced by the presence of anticomplementary substances which mask the full activity of cord serum. However, the possibility of complement synthesis by the fetus has not been ruled out. The results of the present studies, although not warranting definite conclusions on either passage or synthesis, tend to support the former concept. In terms of total activity, ratios of cord to maternal sera were consistently similar. Within paired specimens, the ratio of individual cord complement components to corresponding maternal com-

TABLE II. Total Complement Hemolytic Activity of Equal Volume Mixtures of Maternal and Cord Sera.

| | Complement titer (C′H50 units/ml) | | | |
|---|---|---|---|---|
| Specimens | Cord | Maternal | Mixture | Expected |
| C 14 + M 13 | 50 | 109 | 79.0 | 79.5 |
| C 14 + M 14 | 50 | 93 | 66.0 | 71.5 |
| C 15 + M 13 | 60 | 109 | 75.5 | 84.5 |
| C 15 + M 14 | 60 | 93 | 72.0 | 76.5 |
| C 16 + M 16 | 63 | 131 | 70.5 | 95.5 |

TABLE III. Total Protein and Electrophoretic Analyses of Maternal and Cord Sera.

| Sera | Total protein (g Folin Pro-Sol* equiv./100 ml) | Albumin | | α-Globulin | | β-Globulin | | γ-Globulin | |
|---|---|---|---|---|---|---|---|---|---|
| | | %† | g % | % | g % | % | g % | % | g % |
| M 3 | 7.8 | 56 | 4.36 | 15.7 | 1.23 | 16.8 | 1.31 | 11.5 | .90 |
| C 3 | 7.9 | 76 | 6.00 | 4.3 | .34 | 7.9 | .63 | 11.8 | .93 |
| M 5 | 7.7 | 53 | 4.16 | 10.3 | .79 | 23.8 | 1.83 | 12.9 | .99 |
| C 5 | 6.4 | 78 | 5.00 | 5.3 | .34 | 7.0 | .48 | 9.7 | .62 |
| M 6 | 7.4 | 49 | 3.62 | 16.5 | 1.22 | 22.5 | 1.66 | 12.0 | .89 |
| C 6 | 7.4 | 61 | 4.46 | 10.8 | .80 | 13.1 | .97 | 15.6 | 1.15 |
| M 7 | 8.8 | 42 | 3.70 | 18.5 | 1.62 | 21.6 | 1.90 | 17.9 | 1.57 |
| C 7 | 6.7 | 55 | 3.68 | 8.9 | .60 | 12.9 | .87 | 23.6 | 1.58 |
| M 10 | 8.4 | 52 | 4.40 | 15.4 | 1.29 | 18.6 | 1.56 | 13.5 | 1.13 |
| C 10 | 7.5 | 66 | 4.81 | 8.6 | .64 | 11.5 | .86 | 14.9 | 1.11 |
| M 11 | 6.6 | 34 | 2.24 | 23.0 | 1.52 | 30.0 | 1.98 | 13.0 | .86 |
| C 11 | 7.2 | 61 | 4.39 | 9.4 | .67 | 12.2 | .88 | 17.4 | 1.25 |
| M 12 | 9.0 | 45 | 4.05 | 15.0 | 1.35 | 22.4 | 2.02 | 17.6 | 1.58 |
| C 12 | 6.2 | 65 | 4.03 | 7.3 | .45 | 8.4 | .52 | 18.3 | 1.13 |

* Pro-Sol—standard protein solution obtained from Bernhard-Scher Co., New Jersey.
† % obtained from analytrol (see text).

ponents corresponded to the ratio of total activity. If the titer of cord blood represented the activity of components synthesized, it seems likely that the component ratio would exhibit greater variation than that found with one or more of the constituents possibly affected. To our knowledge, these represent the first attempts to examine the decreased level of cord complement activity in terms of component parts.

Although the present data do not support the suggestion that full complement activity of cord serum is reduced by the presence of anticomplementary material, such a possibility cannot be ruled out. Delaying the removal of serum from the clot may actually have reduced such a substance as described by Nattan-Larrier *et al.*(1). If so, inactivation of complement under the conditions of our experiments must have occurred at a greater rate, since Cb titers (Table I) were less than Ca. In addition, the failure of cord serum to reduce the complement level of maternal serum (Table II) could result from complete utilization of an inactivating substance by the cord specimen, leaving none to be active on maternal blood. However, the relatively consistent differences in the paired sera suggest that factors other than anticomplementary activity determine the level of complement in cord sera.

Consistent differences between mother and cord serum specimens were also noted in their electrophoretic patterns (Table III). These observations are in complete agreement with those of Moore *et al.*(11). The major variation appeared in the alpha- and beta-globulin region with the maternal samples containing a higher concentration of both fractions. The migration of complement within this region and the lower activity of cord sera may be related to the deficiencies in concentration of the alpha- and beta-globulins in cord serum. The fact that the fetal liver and placenta can produce these proteins(12), lends indirect evidence supporting the concept of complement synthesis. More study is necessary before a final conclusion concerning the origin of this substance in cord blood can be made.

*Summary.* Complement hemolytic activities of sera, obtained from umbilical cords at birth, were compared to those obtained from the corresponding mothers. The lower total activity of the cord specimens was correlated with a parallel decrease in all components. Anticomplementary substances could not be detected in cord samples. The reduced concentration of complement components in cord sera was correlated with reduced levels of alpha- and beta-globulins as determined by paper electrophoresis. It may be more than coincidental that 3 of the 4 components also migrate in this region.

The authors wish to thank Drs. D. W. Talmage and Richard Thompson for their suggestions, and Dr. Roger Cadol, Carolyn Keegan, and Melvin Britton for collection of blood samples.

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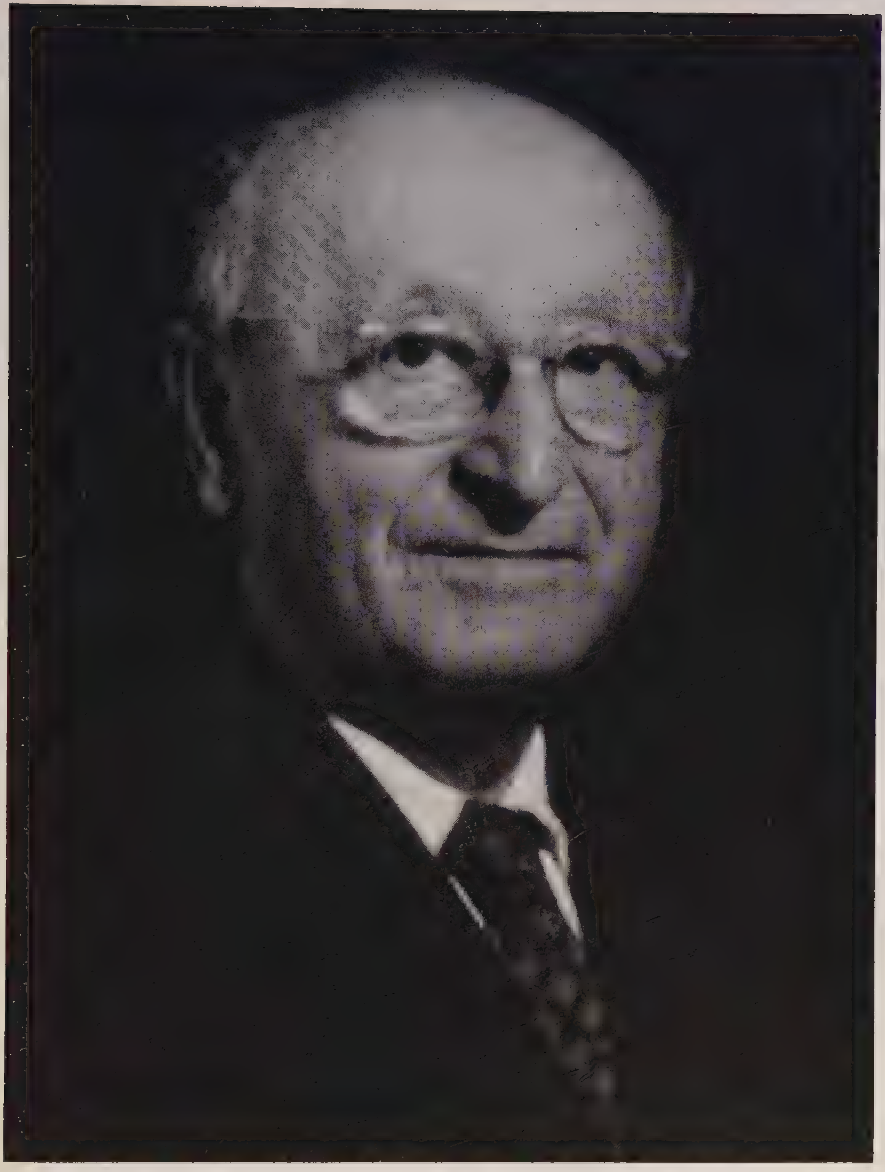

Dr. A. J. Goldforb, who retired as Secretary-Treasurer and Managing Editor of the Society for Experimental Biology and Medicine on July 1, 1960, was born in London, England, March 13, 1881. He came to the United States in 1884; received his B. S. degree from the College of the City of New York in 1900, and his Ph.D. from Columbia University in 1909. He was a member of the faculty of the City College from 1910, becoming an emeritus professor in 1951. His publications were in the fields of experimental embryology and general physiology.

Dr. Goldforb is a member of the following learned societies: American Naturalists; American Society of Zoologists; Society for Experimental Biology and Medicine; American Physiological Society; New York Academy of Medicine; Marine Biological Laboratory, Woods Hole, Mass.; Marine Biological Laboratory, Bermuda; Sigma Xi.

Dr. Goldforb became a member of the Society for Experimental Biology and Medicine in 1909. He became its Secretary-Treasurer and Managing Editor in 1924, and served in these capacities until his retirement in 1960. His history of the Society which appears on the following pages indicates the growth of the prestige of the Society as well as the increasing number of contributions to science published in the Proceedings during the many years when the Society was subject to his wise leadership.

# A Brief History of the Society for Experimental Biology and Medicine

A. J. GOLDFORB

*Past Managing Editor and Secretary-Treasurer (1924-1960)*

IN 1923 the officers of the Society invited me to accept the offices of Secretary-Treasurer and Managing Editor. They pledged to share in the work and stated that my duty was to apportion the tasks and to coordinate their activities.

After 37 years service I requested the Council to be relieved of my responsibilities. The Council asked me to write a brief history of the Society, to be published in our *Proceedings*. The following is in accordance with their unanimous request.

## FOUNDING OF THE SOCIETY

It should be recalled that prior to the founding of the Society, biology and scientific medicine were essentially morphologic, descriptive or systematic sciences. Germany led the world in the use of experimental methods in the solution of problems in these sciences. Talented workers from practically all advanced countries flocked to Germany to learn the new experimental methods.

Dr. S. J. Meltzer of Russia was one of the early migrants to Berlin in 1876. There he studied under the great leaders, *viz.*, Professors DuBois-Raymond, Virchow, Frerich, Leyden, Ludwig, Helmholz, Koch and Kronecker. In 1883 he migrated as Ship Doctor to New York City. There he was associated with the then leaders in medical science, including Drs. S. Flexner, Howell, Janeway, Jacobs, Lusk, Park, Prudden, Welch, Jacobi, Knopf and Osler.

Dr. Meltzer was soon busy in the practice of medicine. His physiologic researches were continued in the basement of his home. His other activities included founding of the Society for Experimental Biology and Medicine and later the American Association of Clinical Research or "Young Turks." He was one of the leaders in the Association of American Physicians, the American Physiological Society and the Harvey Society. Late in life he became a Member of the Rockefeller Institute, his first research post.

On January 17, 1903, Dr. Meltzer invited a small group of leaders in biologic and medical sciences in New York City to consider (1) the advisability of founding a society where researches in experimental biology and experimental medicine, *as one science,* were to be reported, (2) where *younger scientists* were to be stimulated to use experimental and controlled methods in the solution of their problems, and (3) where the *highest possible standards* should prevail. The group unanimously approved the plan and became charter members of the Society. They were Drs. G. N. Calkins, H. E. Crampton, E. B. Wilson (biologists), R. H. Cunningham, J. Ewing, W. J. Gies, C. A. Herter, P. H. Hess, H. C. Jackson, F. S. Lee, P. A. Levine, G. Lusk, S. J. Meltzer, C. Norris, R. Burton-Opitz, W. H. Park, A. N. Richards, P. G. Stiles, and G. B. Wallace (medical scientists).

Five meetings were held in the first academic year, at which each member reported or demonstrated his experiments. Practically every one attended each meeting and critically discussed each report or demonstration. These meetings were intimate. They were held in Bellevue Medical College and in the College of Physicians and Surgeons of Columbia University.

A few *non-resident* scientists were elected to membership. Resident *non-members* were invited to report their findings, and if the report was of high merit, the author was elected to membership. At the end of the first academic year there were 19 charter members, 14 elected resident members and 22 non-resident members from the Universities of Pennsylvania, Yale, Johns Hopkins and Harvard. The total membership was 55.

It was decided to publish *very brief abstracts* of papers read at the meetings. These appeared as the *Proceedings of the Society*, published during the first three years in *Science* and in *American Medicine.* With 55 members in the first year, 61 abstracts were published and with indices constituted 75 pages of Vol. 1. There was prompt and widespread interest in the Society and its Proceedings.

On November 7, 1920, Dr. Meltzer died. A memorial meeting was held January 6, 1921, jointly arranged by the New York Academy of Medicine, the Harvey Society, and the Society for Experimental Biology and Medicine. The meeting was addressed by Drs. W. H. Howell (physiologist, Johns Hopkins), G. Calkins (biologist, Columbia University), P. A. Levine (chemist, Rockefeller Institute), G. Lusk (physiologist, Cornell Medical College), G. B. Wallace (pharmacologist, New York University Medical College), W. Welch (pathologist, Johns Hopkins). The speakers extolled the role of Dr. Meltzer as the distinguished leader in the United States in promoting the use of experimental controlled methods in the solution of biological and medical problems.

### HISTORY OF THE "PROCEEDINGS"

These were published during the first 3 years in *Science* and in *American Medicine*. Beginning with the fourth year they were published by the Society, at Dr. Gies's expense.

With rapid growth in membership and in number of manuscripts, the *volumes published per year* were correspondingly increased from one (1903-1934) to 3 (1938-1960) (Table 1, column 2). The *number of articles* published rose rapidly from 61 in 1904 to 894 in 1959, a 14-fold increase (Table 1, column 3). The *number of monthly issues* a year rose from 5 in 1914 to 11 in 1949 (Table 1, column 4). The *number of pages* increased much faster, *viz.*, from 75 in 1904 to 2659 in 1959, a 35-fold increase (Table 1, column 5).

*Length of articles* also increased. In 1903-4, *92% of the articles were 1/10 to one printed page*, single column and large type, with few brief tables and illustrations. The per cent of such short papers progressively *decreased to 0.5%* in 1958 (Table 2). *Two page* articles increased steadily from 7% in 1904 to 51% in 1932-8. *Three page articles* increased from 1% in 1903-4 to 37% in 1958. Four page articles increased from 0% in 1903-4 to 23% in 1958, etc. (Table 2). Beginning in 1942, articles were published in double column and in smaller type, so that a page contained 1.6 times as many words. Hence, length of articles *was over 1.8 times longer* than indicated in Table 2.

In view of the above progressive increases, the following plans were adopted. (1) Until 1924 all articles were "Preliminary." Thereafter the author labelled the manuscript as either "Preliminary"(P) or "Complete"(C). For reasons given below, the number of P articles steadily decreased from 100% (1904 to 1924) to 20% in 1949 (Table 1, column

TABLE 1. Progressive Increase in Number of Volumes, Issues, Articles, Pages and Editors on Successive 5 Years.

| 1<br>Year | 2<br>Vol/yr | 3<br>Art./yr published | 4<br>Issues/yr | 5<br>Pages/yr | 6<br>No. of editors | 7<br>% prelim. art. | 8<br>No. of members |
|---|---|---|---|---|---|---|---|
| 1903- 4 | 1 | 61 | 5 | 75 | 0 | 100 | 56 |
| 1908- 9 | 1 | 72 | 5 | 158 | 0 | 100 | 185 |
| 1913-14 | 1 | 115 | 6 | 219 | 0 | 100 | 274 |
| 1918-19 | 1 | 184 | 6 | 208 | 0 | 100 | 379 |
| 1923-24 | 1 | 291 | 8 | 652 | 3 | 100 | 627 |
| 1928-29 | 1 | 476 | 8 | 930 | 8 | 60 | 986 |
| 1933-34 | 1 | 563 | 9 | 1342 | 11 | 54 | 1218 |
| 1938-39 | 3 | 788 | 9 | 1908 | 15 | 48 | 1488 |
| 1943-44 | 3 | 538 | 9 | 949 | 21 | 17 | 1774 |
| 1948-49 | 3 | 718 | 11 | 2146 | 23 | 20 | 2388 |
| 1953-54 | 3 | 744 | 11 | 2381 | 31 | 0 | 2929 |
| 1958-59 | 3 | 894 | 11 | 2659 | 37 | 0 | 3336 |

TABLE 2. Increasing Length of Articles (in %) in Successive 5 Years.

| | 1903-4 S | 1907-8 S | 1913-14 S | 1922-28 S | 1932-38 S | 1945-46 D | 1957-58 D |
|---|---|---|---|---|---|---|---|
| To 1 page | 92 | 54 | 50 | 40 | 20 | 23 | 0.5 |
| 2 | 7 | 30 | 43 | 33 | 51 | 32 | 22 |
| 3 | 1 | 8 | 7 | 15 | 15 | 20 | 37 |
| 4 | 0 | 6 | 1 | 5 | 6 | 10 | 23 |
| 5 | 0 | 0 | 0 | 2 | 6 | 8 | 11 |
| 6 | 0 | 1 | 0 | 1 | 1 | 3 | 4 |
| 7 | 0 | 0 | 0 | 1 | 1 | 2 | 1 |
| 8 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |

S = single column per page, large type. D = double column per page, small type.

7.) Beginning in 1923 *Editors were appointed* (Table 1, column 6). *Standards of acceptance* by the Editors were progressively raised. For *Preliminary articles* an *adequate statement of methods* was required. An illustrative experiment(s) was to be given in detail. Variability of results was required. Conclusions must be based on experimental data submitted. Standards for *Complete articles* were likewise raised.

To *meet these higher standards,* the maximum of 3 printed pages, especially for Complete articles, was increased to 5, then to 8 and finally to 10 printed pages, including Tables, illustrations and bibliography. This last maximum was equivalent in number of words to the 15-page maximum of other journals. It may be of interest to note that many authors interpreted the above changes to mean that the author was expected to inflate his manuscript to the new maximum. The Editors then were forced to ask for more and more condensation of articles.

## THE BOARD OF EDITORS

### *Increase of Editors and Higher Standards*

Until 1923 there was no Board of Editors. As the membership increased rapidly, there was a lowering of standards of manuscripts received. The Council then voted to establish a Board of Editors. The Editors determined whether a manuscript met the new standards, whether it should or should not be published, and suggested the necessary changes. The first Editorial Board included Drs. H. Jackson, W. MacNider, A. M. Pappenheimer, and the Managing Editor. Much time was spent in planning editorial policies. The number of editors was progressively increased to 37 in 1960 (Table 1, column 6). This enlargement of the Board provided for editors in different sub-specialties of a major science. For example, in the major field of bacteriology there were 7 editors, specialists in virology, infectious diseases, antigens, immunology, antibiotics, microbiology or general bacteriology. A manuscript could now be sent to editors in related fields. Editors in each group received approximately the same number of manuscripts each year. Table 3 is our Honor Roll of Editors who served three or more years. The Table includes dates of service.

### *Problem of Wide Variation Among Editors in Each Group*

Editors' standards were measured by (1) % of mss. rejected by each editor in the group; (2) % returned for radical revision; (3) % returned for major changes; (4) % returned for radical condensation; (5) % of new manuscripts approved; (6) % of revised manuscripts approved, etc. Table 4 gives such data for 1958-59.

Using these and other data for judgment of an editor's standards, it was found that many newly elected editors tended to be either oversevere *i.e.*, to 91% rejected, or overlenient. Some made every effort to help an author, some did not. Some readily admitted when they made an error of judgment, others did not do so. Some approved most revised manuscripts, others did not.

Table 4 gives some of the data for 1958-59. In Group 1, for example, there were 7 editors. The per cent of new manuscripts rejected varied in this group from 7 to 24%. The per cent of manuscripts returned for radical revision ranged from 0 to 14. Similar variation occurred for manuscripts returned

TABLE 3. Honor List of Editors Who Served Three or More Years (to 1959)

| | | | | | |
|---|---|---|---|---|---|
| MacNider, W. B. | 1923-29 | Werkman, C. H. | 1945-50 | Haurowitz, F. | 1954-60* |
| Pappenheimer, A. M. | 23-29 | Allen, W. M. | 46-51 | Mackenzie, C. G. | 54-56 |
| Sherman, H. C. | 24-28 | Berg, C. P. | 46-51 | McCarty, M. | 54-59 |
| Carlson, A. J. | 27-35 | Kleitman, N. | 46-51 | Syverton, J. T. | 54-58 |
| Duggar, B. M. | 27-31 | Shope, R. E. | 46-51 | Winzler, R. | 54-58 |
| Jacobs, M. B. | 27-34 | Dempsey, E. W. | 47-52 | Witschi, E. | 54-59 |
| Kollmer, J. A. | 27-31 | Goodman, L. S. | 47-52 | Habel, K. | 54-60* |
| Lewis, H. B. | 28-37 | Horsfall, F. L. | 47-52 | Hoff, H. E. | 55-60 |
| Bayne-Jones, S. | 32-34 | Potter, V. R. | 47-52 | Johnson, B. C. | 55-60* |
| Murlin, J. R. | 32-34 | Warren, S. | 47-52 | Leathem, J. H. | 55-60* |
| Williams, H. S. | 32-36 | Burk, D. | 48-53 | Warner, E. D. | 55-59 |
| Bronk, D. W. | 35-40 | Eagle, H. | 48-53 | Almquist, H. J. | 56-60* |
| Corner, G. W. | 35-38 | Loew, E. R. | 48-53 | Axelrod, A. E. | 56-60* |
| Hastings, A. B. | 35-40 | Angevine, D. | 49-54 | Dougherty, T. F. | 56-58 |
| Hooker, S. B. | 35-41 | Dingle, J. H. | 49-54 | Krampitz, L. | 56-60 |
| McCann, W. S. | 35-38 | Quigley, J. P. | 49-54 | Pappenheimer, J. | 56-60 |
| Rivers, T. M. | 35-41 | Richardson, A. P. | 49-54 | Samuels, L. T. | 56-59 |
| Danforth, C. H. | 36-41 | Wells, J. A. | 49-54 | Schueler, F. W. | 56-60* |
| Ivy, A. C. | 36-41 | Brinkhous, K. M. | 50-55 | Schwerdt, C. | 56-60 |
| Plant, O. H. | 36-38 | Coggeshall, L. T. | 50-55 | Anderson, E. | 57-60* |
| Rich, A. R. | 37-43 | Dunn, M. S. | 50-55 | Coons, A. H. | 57-60 |
| Wilson, D. W. | 38-41 | Gray, J. S. | 50-55 | Ginsberg, H. S. | 57-60* |
| Geiling, E. M. K. | 39-43 | Heinle, R. W. | 50-55 | Jailer, J. W. | 57-60 |
| Marshall, E. K. | 39-43 | Loosli, J. K. | 50-55 | Liebow, A. | 57-60 |
| Tillet, W. S. | 39-43 | Smith, E. L. | 50-55 | Mehl, J. W. | 57-60* |
| Wintersteiner, O. | 39-43 | Umbreit, W. W. | 50-55 | Schlumberger, H. G. | 57-60 |
| Bieter, R. N. | 40-43 | Acheson, G. W. | 51-55 | Stokstad, E. L. R. | 57-60* |
| Dubos, R. J. | 40-43 | Moore, C. R. | 51-54 | Wright, L. D. | 57-60 |
| Elvehjem, C. A. | 40-43 | Stanley, W. M. | 51-56 | Benditt, E. P. | 58-60* |
| Mann, F. C. | 40-43 | White, A. | 51-56 | Borison, H. L. | 58-60* |
| Mattill, H. A. | 40-43 | Astwood, E. B. | 52-56 | Graham, J. B. | 58-60* |
| Engle, E. T. | 41-46 | Furth, J. | 52-57 | Hollaender, A. | 58-60* |
| Francis, T., Jr. | 41-43 | Kidd, J. G. | 52-58 | Jawetz, E. | 58-60* |
| Gerard, R. W. | 41-43 | Nickerson, M. | 52-57 | Miller, L. L. | 58-60* |
| Sendroy, J., Jr. | 41-48 | Raffel, S. | 52-57 | Zucker, M. B. | 58-60* |
| Shannon, J. A. | 41-43 | Sayers, G. | 52-57 | Brobeck, J. R. | 59-60* |
| Long, C. N. H. | 42-47 | Riker, W. F. | 53-56 | Engel, F. L. | 59-60* |
| Dragstedt, C. A. | 43-48 | Bain, J. A. | 54-59 | Grossman, M. I. | 59-60* |
| Crandall, L. A. | 44-49 | Bernheim, F. | 54-57 | Harrington, W. J. | 59-60* |
| Seevers, M. H. | 44-49 | Boyer, P. D. | 54-57 | Maren, T. | 59-60* |
| VanDyke, H. B. | 44-49 | Davenport, H. | 54-60 | Rogers, S. | 59-60* |
| Greenberg, D. M. | 45-50 | Farah, A. | 54-59 | Sawyer, C. H. | 59-60* |
| Norris, L. C. | 45-50 | Follis, R. H., Jr. | 54-59 | Unna, K. R. W. | 59-60* |
| | | | | Wannamaker, L. W. | 59-60* |

* Continuing as Editor.

for major changes or for sharp condensation. The per cent of new approved manuscripts ranged from 43 to 78%, for revised manuscripts 10 to 30%. The per cent of rejected manuscripts in Group I ranged from 7 to 24%, in Group II, 9-31%, Group III, 8 to 44%, Group IV, 7 to 53%, etc. Table 4 also shows the wide variation for manuscripts marked by Editors as "Excellent," "Good," or "Passable." In Group I, for example, "Excellent" manuscripts ranged from 4 to 50%, "Good" manuscripts from 42 to 75%, "Passable" manuscripts from 7 to 42%. Similar wide variations occurred in the other groups of editors. No one item is convincing, but all items give a fair indication of an editor's standards.

*To reduce this wide variation of editors' standards* in each group, manuscripts were frequently referred to more than one editor, *i.e.*, from an editor of low standards to one of high standards, and vice versa. The task of a Managing Editor, with such wide variation, is not an enviable one.

When an editor was in doubt whether a manuscript was acceptable, it was referred to other editors. With a manuscript in a highly specialized field, the editor might refer it to an outstanding non-editor, *i.e.*, to a referee. Our experience with referees has,

TABLE 4. Variation in Standards within Each Group of Editors for 1958-59.†

| (1) Groups of editors | (2) Editors No. | (3) % rej.* | (4) % rad.* | (5) % approved New mss. | (6) % approved Revised mss. | (7) % marked by editors as Excellent | (8) Good | (9) Passable | (10) Doubtful |
|---|---|---|---|---|---|---|---|---|---|
| I | 1 | 7 | 4 | 78 | 10 | 50 | 42 | 8 | 0 |
| | 2 | 24 | 6 | 49 | 21 | 11 | 44 | 42 | 3 |
| | 3 | 18 | 14 | 43 | 25 | 26 | 63 | 7 | 0 |
| | 4 | 20 | 5 | 45 | 30 | 6 | 75 | 15 | 3 |
| | 5 | 18 | 0 | 53 | 29 | 11 | 52 | 37 | 0 |
| | 6 | 17 | 6 | 66 | 11 | 4 | 70 | 26 | 0 |
| | 7 | 8 | 8 | 50 | 30 | 16 | 45 | 39 | 0 |
| II | 1 | 24 | 3 | 59 | 14 | 19 | 69 | 12 | 0 |
| | 2 | 19 | 3 | 55 | 22 | 21 | 64 | 13 | 1 |
| | 3 | 9 | 12 | 57 | 21 | 9 | 71 | 20 | 0 |
| | 4 | 25 | 6 | 41 | 28 | 26 | 41 | 26 | 6 |
| | 5 | 30 | 15 | 26 | 29 | 4 | 36 | 43 | 17 |
| | 6 | 31 | 15 | 34 | 19 | 11 | 67 | 16 | 4 |
| | 7 | 15 | 9 | 44 | 32 | 3 | 77 | 20 | 0 |
| III | 1 | 17 | 5 | 59 | 20 | 11 | 49 | 40 | 0 |
| | 2 | 10 | 13 | 33 | 43 | 0 | 68 | 32 | 0 |
| | 3 | 8 | 18 | 31 | 43 | 0 | 53 | 36 | 10 |
| | 4 | 19 | 30 | 12 | 40 | 5 | 64 | 19 | 12 |
| | 5 | 44 | 0 | 32 | 24 | 4 | 79 | 14 | 3 |
| IV | 1 | 53 | 26 | 5 | 16 | 0 | 50 | 50 | 0 |
| | 2 | 7 | 21 | 36 | 36 | 19 | 69 | 12 | 0 |
| | 3 | 15 | 4 | 41 | 39 | 16 | 47 | 26 | 10 |
| | 4 | 12 | 28 | 20 | 40 | 7 | 43 | 40 | 10 |
| V | 1 | 24 | 17 | 31 | 27 | 0 | 52 | 36 | 12 |
| | 2 | 19 | 29 | 13 | 39 | 13 | 42 | 35 | 10 |
| | 3 | 34 | 15 | 19 | 31 | 0 | 28 | 62 | 9 |
| | 4 | 44 | 13 | 13 | 30 | 4 | 64 | 8 | 24 |
| VI | 1 | 18 | 18 | 36 | 29 | 14 | 58 | 23 | 5 |
| | 2 | 33 | 6 | 50 | 11 | 6 | 31 | 56 | 6 |
| | 3 | 37 | 17 | 26 | 19 | 11 | 52 | 20 | 16 |
| VII | 1 | 27 | 9 | 46 | 17 | 27 | 50 | 17 | 6 |
| VIII | 1 | 14 | 16 | 34 | 36 | 6 | 74 | 20 | 0 |
| Total | 32 | | | | | | | | |

* Rej. = rejected mss. Rad. = radical revision.
† % important changes and sharp condensation not listed.

with exceptions, not been a happy one. If the referee belongs to one school of thought and is opposed to other schools, is he an impartial referee? Does the referee have a personal antagonism to the author, or does he favor the author? Is such a referee a qualified arbiter? Does he try to help the author or are his comments perfunctory? Unless we know such attributes of the referee should one send him manuscripts for his judgment?

Editors met annually to discuss editorial policies and to consider new problems and higher standards. The record of each editor was considered. As a result there was a definite trend for over-severe editors to conform with the group and for over-lenient editors to raise their standards.

*Authors' Standards* also varied widely. A good manuscript of substance and well written is a labor of love to process for the printer. Too often the manuscript is carelessly written. It is either a technician's report of unorganized laboratory notes, or the author cannot or will not write understandably, or he does not consider it necessary to mention controls, or he draws conclusions not based on the evidence. Some expect the Editor to recalculate the data, to reorganize or rewrite the manuscript, or to supply necessary bibliography. When a conscientious editor does these things he is likely to be roundly scolded by the author. An instance of another sort may be of interest. The editors recommended *at least 50% reduction.* The authors returned the manuscript with their thanks to the editors, and stated that they had *complied precisely* with their suggestions. The original manuscript was 12 pages long, the revised manuscript 6 pages. The original, however, was *double spaced,* and in large type, the revised manuscript *single space* in small type. *Not a word had been deleted.* He expressed surprise when the revised manuscript was promptly returned to him. Manuscripts are not infrequently returned to the author for important changes, not once, but 2 to 6 times.

### THE PROBLEM OF SPONSORED ARTICLES

During the early history of the Society, members knew each other well. With rapid increase in membership and with increasing Sections in distant areas, there was less and less personal knowledge of members and of applicants for membership. More and more non-members submitted manuscripts, particularly from abroad. There occurred a lowering of standards of manuscripts and of applicants for membership, and *also greater irresponsibility of sponsors. They sponsored articles unread,* or not critically read, or they sponsored manuscripts of authors unknown to them, or sponsored articles identical with those previously published in 2 to 5 other journals.

The per cent of sponsored articles sharply increased. By 1959 49% of articles published in the Proceedings, were sponsored. The per cent of sponsored articles rejected or returned for radical revision, etc., was far greater than for non-sponsored articles.

Hence the Editorial Board requested the following changes in policy.

(1) Members should not sponsor a manuscript by an author unknown to them, or who had done his researches in another institution. Exception may be made if author and sponsor had previously worked in the same laboratory. The sponsor should state how well and how long he has known the author as a scientific worker.

(2) The sponsor should write as to the author's scientific integrity.

(3) The sponsor should *critically* examine the manuscript and state whether it conforms with our "Suggestions to Authors."

(4) He should also state wherein a significant contribution to science was made.

These rules should be assumed. When more sponsors cooperate these rules should be discarded.

### THE PROBLEM OF INCREASING MEMBERSHIP

In the early history of the Society all members were leaders in biology or medicine. They demanded very high standards of applicants to membership, and were exceedingly careful concerning sponsoring of articles. As the membership rapidly grew, standards frequently dropped.

In the first year (1903-4) there were 19 charter members and 36 newly elected members, total 55. At the end of the second year there were 42 resident members and 45 non-resident members, total 87. By 1907 there were 59 resident and 93 non-resident members from Alabama 1, California 5, Connecticul 6, D. C. 4, Illinois 11, Maryland 16, Massachusetts 16, Michigan 6, Missouri 1, New York State 6, Ohio 3, Pennsylvania 6, Wisconsin 6, total 152. The increase in membership in successive 5 years is shown in Table 1, column 8. The increase was from 55 in 1904 to 3336 in 1959, an increase of 60-fold. The Council in due time voted that a *National Membership Committee* of three be set up, primarily to raise the standards of acceptance of applications. This Committee was subsequently enlarged to 5, later to 9 (in 1929), including a member from nine *different* Sections, each nominated by the Section. In the early years a unanimous vote of the Committee was required for acceptance. Later a 2/3 favorable vote of the Committee sufficed. Doubtful applications were reconsidered at the annual meeting of the Committee. Each member of the Committee served 4 years (except the Chairman).

Each application was sponsored by three members of the Section and endorsed by the Section Membership Committee before consideration by the National Membership Committee.

But the records showed that sponsors, Section Membership Committee, and the National Membership Committee tended more and more to be "kind-hearted" or ultra "democratic" by approving an increasing per cent of applications even where eligibility was very, very doubtful. In 1959 the National Membership Committee received 141 applications. Their vote was as follows: Member No. 1 approved 81%; No. 2, 79%; No. 3, 61%; No. 4, 49%; No. 5, 49%; No. 6, 43%; No. 7, 32%; No. 8, 26%; and No. 9, 14%. Nos. 1 and 2 were practically non-selective. They were Section advocates. No. 3 approved 2/3 of the applicants, Nos. 4, 5 and 6 approved about ½, Nos. 7, 8 and 9 approved ⅓ to ⅙ of the applicants. These last were the selective members, but were outvoted. These results were fairly typical for later years.

The Council then voted that a committee of the Council, *viz.* the President, Vice-President, General Secretary, and 2 Councillors-at-large be requested to review applications concerning which there was doubt as to acceptability, even though approved by the Section Membership Committee and the National Membership Committee. All correspondence was sent with each application. Forty such applications were sent to this Council Committee for review. They reported as follows: *45% were rejected, 32% were referred back* to sponsors or Section for further information. *Only 22% were approved.* One member of the Council Committee voted consistently for "democratic" acceptance of practically all applicants, except those referred back for further information.

The problem is, what may be done to raise standards of acceptance of applications for membership, and *therefore of their articles and articles sponsored by them.*

*Associate Members.* To encourage younger scientists, not yet eligible to membership, and to encourage non-experimental workers to take part in the activities of the Section, provision was made for their election *by the Section,* as associate members of the Section.

*Foreign Members.* Eligible scientists outside the United States and Canada could be elected to membership. In 1957 there were at least 113 foreign members.

*Emeritus Members.* A member who retires from his post, by age or serious illness, may be transferred by the Council to emeritus membership. He then pays no dues. In 1959 there were at least 218 emeritus members.

## FORMATION OF SECTIONS

Originally members were all from New York City. Subsequently non-residents were elected. As the number of non-residents increased, Sections were authorized. When 20 or more members in an area requested that a Section be formed and gave the name of the proposed Section, the geographical boundaries, the consent of officers of adjoining Section(s) (if any), and a statement of conformity with our constitution and by-laws, the Council approved such request. Later the required number of petitioners was increased to 30. By 1959, 21 Sections were approved. Two withdrew, *viz.,* Peiping in 1952 and Massachusetts in 1927. The list of Sections and date of admission is shown in Table 5.

*Section Activity and Section Standards* may be measured by (1) the number of Section meetings per year; (2) number of members at these meetings; (3) number of manuscripts received and the per cent approved; (4) attendance of Chairman, Secretary, and member (if any) of the National Membership Committee at annual meetings; (5) number of applications for membership and per cent approved.

Table 6 gives the number of Section meetings in successive 5 years. This number varies

TABLE V. List of 19 Sections and Date when Authorized.

| Section | Date when authorized | Section | Date when authorized |
|---|---|---|---|
| New York | 1903 | So. California | 1933 |
| Pacific Coast | 1920 | Dist. Columbia | 1934 |
| Minnesota | 1921 | Rocky Mountain | 1941 |
| Western N.Y. | 1921 | Southwestern | 1944 |
| Missouri | 1925 | Maryland | 1947 |
| Illinois | 1926 | Ohio Valley | 1947 |
| Iowa | 1926 | Southeastern | 1948 |
| Southern | 1926 | Michigan | 1958 |
| Wisconsin | 1927 | Northwest | 1958 |
| Cleveland | 1930 | | |

TABLE 6. No. of Meetings per Section for Successive 5 Years.*

| Year | Cleveland | Dist. Columbia | Illinois | Iowa | Maryland | Michigan | Minnesota | Missouri | New York | Northwestern | Ohio Valley | Peiping | Pac. Coast | Rocky Mt. | Southeastern | Southern | S. California | Southwestern | Western N.Y. | Wisconsin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1904-14 | | | | | | | | | 5-8 | | | | | | | | | | | |
| 1919 | | | | | | | | | 8 | | | | 3 | | | | | | | |
| 1924 | | | | | | | 6 | | 8 | | | 6 | 4 | | | | | | 5 | |
| 1929 | | | 4 | 3 | | | 4 | 5 | 8 | | | 3 | 5 | | | 3 | | | 4 | 1 |
| 1934 | 4 | | 3 | 3 | | | 5 | 5 | 8 | | | 1 | 4 | | | 3 | | | 4 | 0 |
| 1939 | 7 | 3 | 4 | 3 | | | 5 | 5 | 5 | | | 3 | 4 | | | 4 | 4 | | 4 | 1 |
| 1944 | 4 | 4 | 3 | 4 | | | 3 | 1 | 3 | | 0 | 0 | 5 | 3 | | 2 | 3 | | 0 | 0 |
| 1949 | 7 | 0 | 4 | 0 | 1 | | 6 | 3 | 2 | | 2 | 1 | 6 | 3 | 1 | 3 | 4 | 2 | 3 | 2 |
| 1954 | 4 | 5 | 5 | 6 | 3 | | 4 | 4 | 2 | | 1 | | 4 | 1 | 1 | 6 | 0 | 2 | 0 | 0 |
| 1959 | 7 | 3 | 4 | 5 | 3 | 3 | 5 | 1 | 0 | 1 | 1 | | 3 | 0 | 1 | 2 | 3 | 1 | 1 | 1 |
| No. of mss. published in 1958-59 | | | | | | | | | | | | | | | | | | | | |
| | 12 | 69 | 61 | 9 | 22 | 34 | 32 | 23 | 135 | 12 | 50 | | 38 | 5 | 27 | 19 | 45 | 58 | 24 | 10 |

Non-sections, 144 mss. Foreign, 51 mss. Total mss. published, 880

* Blank spaces indicate Section not yet established.

widely in different Sections and varies in a given Section in different years. For example, in 1954 the number of Section meetings per year varied from 0 to 6. In a given Section the number varied in successive 5 years from 0 to 8. The number of manuscripts published per Section in 1958-59 ranged from 5 in the Rocky Mountain Section to 135 in the New York Section. To be sure, these figures should be compared with the number of members in each Section.

Section activity and Section attendance at meetings depends primarily on the interest, ability and energy of the Section Chairman, Secretary and Section Council. When programs of high standards and *broad interest* were arranged, when speakers within or *outside of the Section* were chosen who tell their story effectively and interestingly, when competent scientists were invited to discuss (not perfunctorily), there was no problem of attendance.

Table 7 may be of interest. It gives for 1958-59 the wide variation in different Sections as to (1) Percent of manuscripts rejected, *viz.*, 0 to 33%; (2) % of manuscripts returned for radical revision, 3 to 20%; (3) % of new manuscripts approved for publication, 10 to 58%; (4) % of revised manuscripts approved, 7 to 35%; (5) % of manuscripts rated "Excellent" by the Editors, 0 to 33%; (6) % of "Good" manuscripts, 46 to 78%; (7) % of "Passable" manuscripts, 11 to 43%.

The question arises how to enhance standards in some Sections.

## HISTORY OF NATIONAL OFFICERS AND COUNCIL

The first officers of the Society, in 1903, were a President, Vice-President, Secretary, Treasurer, and Librarian, each elected for one year. The President was Dr. Meltzer, who was reelected for a second term. The Vice-President was Dr. Park. The Secretary was Dr. Gies, who was reelected 6 times. The Treasurer was Dr. Calkins, reelected 5 times. The office of Librarian (Dr. Lusk) was discontinued. The total number of officers was 4 (Table 8).

The by-laws were frequently changed to add Past-Presidents to the list of national officers. There was one past-president in the Council in 1915. The number was progressively increased so that in 1930-37 there were 14 past-presidents on the Council. In 1938 the number was reduced to one, *viz.*, the *last* past-president (Table 8).

In 1916 two *Councillors* were authorized, each to serve one year, later for 2 years, and still later for 4 years. They were elected by vote of the membership and designated Councillors-at-large. In 1958 they were increased to 8, ½ elected every two years (Table 8).

TABLE 7. Shows the Wide Variation in Standards of Manuscripts in 19 Sections for 1958-59.

| Sections | % mss. rejected | % radical revision | % important changes | % sharp condensation | % mss. approved |  | % mss. |  |  |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | New | Revised | Excellent | Good | Passable |
| Cleveland | 13 | 20 | 13 | 27 | 20 | 7 | 9 | 55 | 36* |
| D. C. | 10 | 8 | 15 | 14 | 25 | 27 | 25 | 50 | 25 |
| Illinois | 20 | 11 | 15 | 9 | 14 | 31 | 21 | 50 | 29 |
| Iowa | 33 | 19 | 5 | 0 | 19 | 23 | 0 | 72 | 29 |
| Maryland | 12 | 3 | 1 | 8 | 58 | 18 | 33 | 55 | 11 |
| Michigan | 15 | 5 | 11 | 11 | 34 | 24 | 10 | 78 | 13 |
| Minnesota | 11 | 11 | 11 | 11 | 23 | 31 | 16 | 47 | 37 |
| Missouri | 28 | 12 | 14 | 7 | 19 | 21 | 13 | 55 | 32 |
| New York | 17 | 12 | 10 | 15 | 23 | 22 | 19 | 52 | 30 |
| Northwestern | 0 | 20 | 25 | 10 | 10 | 35 | 0 | 73 | 27* |
| Ohio Valley | 27 | 8 | 9 | 14 | 20 | 22 | 12 | 61 | 27 |
| Pacific Coast | 10 | 10 | 18 | 6 | 32 | 22 | 10 | 68 | 22 |
| Rocky Mt. | 27 | 9 | 18 | 0 | 27 | 19 | 0 | 57 | 43* |
| Southeastern | 18 | 9 | 12 | 18 | 18 | 26 | 5 | 64 | 32 |
| Southern | 15 | 13 | 18 | 8 | 18 | 28 | 7 | 63 | 29 |
| S. California | 19 | 8 | 10 | 10 | 31 | 22 | 3 | 68 | 30 |
| Southwestern | 17 | 13 | 13 | 8 | 17 | 32 | 6 | 63 | 31 |
| Western N.Y. | 17 | 6 | 19 | 15 | 25 | 18 | 3 | 65 | 31 |
| Wisconsin | 5 | 10 | 10 | 15 | 30 | 30 | 15 | 46 | 38* |
| Non-Sect. | 15 | 15 | 13 | 9 | 23 | 23 | 8 | 59 | 34 |
| Foreign | 19 | 12 | 13 | 7 | 27 | 22 | 13 | 60 | 27 |

* Number of mss. few, percentages of doubtful value.

In 1924 the *Chairmen of Sections* automatically became members of the Council. The number of such Chairmen of Sections steadily increased from 0 to 4 in 1924, and to 19 in 1958. (Table 8).

*The Total Number on the Council* grew from 4 in 1903 to 31 in 1958. Thus the changed by-laws provided for frequent rotation of national officers, *viz.*, every 2 years, wide representation, and longer term of office for some officers, *viz.*, Councillors-at-large 4 years, the President 6 years (2 years as Vice-President, 2 years as President and 2 years as Past-President). The Secretary-Treasurer was reelected every two years.

The Council met annually, as did the Board of Editors, the Section Secretaries and the National Membership Committee.

## HISTORY OF OUR FINANCES

It may be of interest to quote the full report of the *first Treasurer* of the Society (Dr. Gies) for the first year, 1903-4, as follows:

| | |
|---|---|
| "Dues received | $16.00 |
| Misc. expenditures | 11.23 |
| Balance on hand | $ 4.77" |

## PRIMARY SOURCES OF INCOME

1. *Membership dues.* In 1903-4 the dues were set at $1 a year. As the Society enlarged and expenditures increased, the dues were raised to $4, $5, $7, $8.50 and $9.50 in 1959 (Table 9). Since members received the Proceedings on payment of dues, and since the dues were below our costs, the increasing membership resulted in a correspondingly greater deficit. (Table 9, columns 1 and 4).

2. *Subscriptions by non-members* began about 1922. In 1924 there were 134 subscribers paying $4 a year. Subscription rates were steadily increased to $6.50 in 1929, to $10 in 1944, and $17 in 1959. In 1959 the income from subscriptions amounted to $51,272, from Dues $31,692, total $82,964. Subscriptions were sold above our costs. Thus the deficit from dues was cancelled *in part* by profits from subscriptions. It is worth noting that the rise in number of subscriptions closely paralled the rise in membership (Table 9, columns 3 and 5).

Costs steadily mounted due to (1) increased number of manuscripts, (2) increased length of articles, (3) greater number and larger Tables and illustrations (which cost 8 to 12 times the equivalent space of text), (3) mounting costs for print-

TABLE 8. Growth and Changes in the Council.

| Year | President | Vice-president | Secretary | Treasurer | Past-pres. | Councillors | Sect. chairmen | Total council |
|---|---|---|---|---|---|---|---|---|
| 1903- 4 | S. J. Meltzer | W. H. Park | W. J. Gies | G. N. Calkins | 0 | 0 | 0 | 4* |
| 1904- 5 | " | J. Ewing | " | " | 0 | 0 | 0 | 4 |
| 1905- 6 | E. B. Wilson | E. Dunham | " | " | 0 | 0 | 0 | 4 |
| 1906- 7 | S. Flexner | " | " | " | 0 | 0 | 0 | 4 |
| 1907- 8 | " | T. H. Morgan | " | " | 0 | 0 | 0 | 4 |
| 1908- 9 | F. S. Lee | " | " | G. Lusk | 0 | 0 | 0 | 4 |
| 1909-10 | " | W. J. Gies | E. L. Opie | " | 0 | 0 | 0 | 4 |
| 1910-11 | T. H. Morgan | " | " | " | 0 | 0 | 0 | 4 |
| 1911-12 | " | P. A. Levine | G. Wallace | " | 0 | 0 | 0 | 4 |
| 1912-13 | J. Ewing | " | " | C. Norris | 0 | 0 | 0 | 4 |
| 1913-14 | " | C. W. Field | H. Jackson | " | 0 | 0 | 0 | 4 |
| 1914-15 | G. Lusk | W. J. Gies | " | J. Murlin | 1 | 0 | 0 | 5 |
| 1915-16 | " | G. Calkins | H. C. Jackson | | 6 | 2 | 0 | 11 |
| 1916-17 | J. Loeb | W. J. Gies | " | | 7 | 2 | 0 | 12 |
| 1918 & 19 | W. J. Gies | J. Auer | " | | 8 | 2 | 0 | 13 |
| 1920 & 21 | G. Calkins | G. Wallace | " | | 9 | 2 | 0 | 14 |
| 1922 & 23 | G. Wallace | J. W. Jobling | " | | 10 | 2 | 4 | 19 |
| 1924 & 25 | H. Jackson | " | A. J. Goldforb | | 10 | 2 | 5 | 20 |
| 1926 & 27 | J. W. Jobling | S. Benedict | " | | 11 | 6 | 8 | 28 |
| 1928 & 29 | S. Benedict | P. Rous | " | | 13 | 6 | 8 | 30 |
| 1930 & 31 | P. Rous | D. Marine & D. Edwards | " | | 14 | 6 | 8 | 31 |
| 1932 | D. Edwards | A. R. Dochez | " | | 14 | 6 | 11 | 34 |
| 1933 & 34 | A. R. Dochez | E. L. Opie | " | | 14 | 6 | 11 | 34 |
| 1935 & 36 | E. L. Opie | P. E. Smith | " | | 14 | 5 | 13 | 35 |
| 1936 & 37 | P. E. Smith | E. R. Dubois | " | | 14 | 4 | 13 | 34 |
| 1938 & 39 | H. Gasser | J. Wearn | " | | 1 | 4 | 13 | 21 |
| 1940 & 41 | J. Wearn | C. D. Leake | " | | 1 | 4 | 14 | 22 |
| 1942 & 43 | W. MacNider | C. Danforth | " | | 1 | 4 | 14 | 22 |
| 1944 & 45 | A. J. Carlson | A. B. Hastings | " | | 1 | 4 | 15 | 23 |
| 1946 & 47 | A. B. Hastings | E. M. Geiling | " | | 1 | 4 | 15 | 23 |
| 1948 & 49 | E. M. Geiling | E. A. Doisy | " | | 1 | 4 | 18 | 26 |
| 1950 & 51 | E. A. Doisy | C. A. Dragstedt | " | | 1 | 4 | 18 | 26 |
| 1952 & 53 | C. A. Dragstedt | C. N. H. Long | " | | 1 | 4 | 18 | 26 |
| 1954 & 55 | C. N. H. Long | T. M. Rivers | " | | 1 | 4 | 18 | 26 |
| 1956 & 57 | T. M. Rivers | W. O. Fenn | " | | 1 | 4 | 18 | 26 |
| 1958 & 59 | W. O. Fenn | P. Bard | " | | 1 | 8 | 19 | 31 |
| 1960 | P. Bard | C. D. Leake | " | | 1 | 8 | 19 | 31 |

* And librarian.

ing, paper, postage, (4) costs of secretarial help and stationery, (5) taxes and insurance etc.

In spite of utmost economies we were forced not only to raise our rates to members and subscribers, but to find other sources of income to meet *some* annual deficits, and unpredictable rising costs. These annual deficits were as follows: 1924, $760; 1925, $420; 1926, $208; 1940, $2676; 1950, $893; 1958, $979.

### ADDITIONAL SOURCES OF INCOME

1. *Space charges.* About 1951 research foundations were asked to share in the costs of printing manuscripts of their grantees.

TABLE 9. Primary Income, *i.e.*, Members' Dues and Subscriptions—5 Year Periods.

| (1) Years | (2) Members' dues | (3) Subscriptions | (4) No. of members | (5) No. of subscriptions |
|---|---|---|---|---|
| 1903-19 | $1.00 | 0 | 55<br>185<br>274<br>379 | |
| 1924 | $1.00 | $ 4.00 | 627 | 134 |
| 1929 | 4.00 | 6.50 | 986 | 365 |
| 1934 | 4.00 | 6.00 | 1218 | 432 |
| 1939 | 4.00 | 6.00 | 1488 | 648 |
| 1944 | 5.00 | 10.00 | 1774 | 1047 |
| 1949 | 7.00 | 13.50 | 2335 | 1495 |
| 1954 | 8.50 | 14.50 | 2929 | 2319 |
| 1959 | 9.50 | 17.00 | 3336 | 3016 |

The then average cost per page was $26. It was suggested that they pay $8 a page, including Tables, illustrations and bibliography. All Foundations and Institutes, except one, cooperated. Special thanks are due to Dr. Shields Warren of the Atomic Energy Commission, for his considerable help in obtaining a revised interpretation of the law, enabling the fiscal officers in U. S. agencies to pay such charges. Our income from such "space charges" permitted us to reduce this charge from $8 to $6.50 a page. In 1959 the income from space charges was $17,019. When a research was not so subsidized no space charge was made. This occurred in less than 2% of the articles.

2. *Advertising*. A professional advertising expert was employed to obtain acceptable advertising. An Advertising Committee was appointed to determine whether the advertising copy submitted was acceptable. Dr. G. W. Cowgill was its chairman. The net income for 1959 was $14,194.

3. *Friends*. In 1940 there was a deficit of $2676. Private Foundations and a select list of pharmaceutical companies were invited to become Friends of the Society, by contributing $150 a year towards publication of the Proceedings. The charge was later reduced to $125. The income for the year 1959 was $4850.

4. *Donors*. There was a deficit in 1950 of $893. In 1951 some Foundations were invited to contribute as Donors. The following contributed:

| | |
|---|---|
| American Cancer Society | $ 400 |
| Damon Runyon Fund | 1000 |
| Griffiths Press | 400 |
| Rogoff Foundation | 1000 |
| Sloan Kettering Institute | 1000. |

Donors were subsequently discontinued.

5. *Reprints*. Originally 25 reprints were given gratis. Additional reprints were sold at printing costs. In 1945 free reprints were discontinued, and costs of reprints included postage and insurance. Later, cost plus 10% profit was charged and finally cost plus 20%. In 1959 the net income from reprints was $2298.

6. *Investments*. Soon after the death of Dr. Meltzer in 1921, his family donated $2000 towards publication costs of the Proceedings. This money was called the *Endowment Fund*. Subsequently it was proposed to increase this Fund by asking our members to contribute $5 a year for three years. Five hundred and fifteen out of 779 members contributed. A Finance Committee of 3 members was appointed to invest our funds conservatively. The Committee discovered that our bankers and brokers gave contradictory advice. Mr. H. G. Friedman, President of General American Investors Corp. was asked to be an unofficial advisor on our investments. He promptly recommended that we dispose of our "guaranteed mortgage bonds." This the Financial Committee did. Soon after came the financial crash in 1929. After the crash, the Society had lost only $2 which is still collectible. In contrast, so many persons and institutions reported frightful losses. Mr. Friedman's second recommendation was to invest our funds in gilt-edge bonds. Later he suggested that a certain per cent be invested conservatively in stocks, as based upon the policies of philanthropies, churches, universities, etc. These recommendations were approved by the Finance Committee and the Council.

Dr. R. A. Lambert suggested that Mr. Edward Robinson, the chief of the investment department of the Rockefeller Foundation, be invited to serve as a second unofficial advisor on investments. The Finance Committee now consists of five persons, *viz.*, Drs. C. A. Doan, C. A. Elvehjem, the Treasurer and two non-member experts, H. G. Friedman and Edward Robinson. The results have been most fortunate. The income from investments for 1958-59 was $6909.

7. *Sale of Back Numbers of the Proceedings*. The annual income varied over the years. In 1959 the income was $2369.

8. *Sustaining Members* voluntarily contributed, usually $3 a year, in addition to dues. This approximated our cost of the Proceedings per member. The income for 1959 was $1066.

9. *Microprint*. By arrangement with the Readex Co., the Proceedings were microphotographed on cards 6 × 9″, each card containing 100 pages of the Proceedings.

The income from the above eight supplementary sources for the fiscal year ending February 28, 1959 was $48,862. The income from dues and subscriptions (primary sources) was for the same year $81,793. The total income was $130,655.

It may be mentioned here that the Secretary-Treasurer and Managing Editor received no salary between 1903 and 1941. The Council urged again and again that he accept a salary and arrange for increased incomes, so that when he retired there would be money to pay his successor. The compromise reached in 1941 was to pay the Secretary-Treasurer and Managing Editor $2500 a year. This was progressively increased by the Council to $6500 in 1959.

*Disbursements* for the year ending February 28, 1959 totalled $111,512. The *surplus* was $19,143. The *net worth* of the Society was $192,907.

*Resumé.* I have outlined (1) the factors that led to the founding of the Society, (2) the policies of the founders, (3) the major changes in policy in the last 57 years.

I have had the privilege of serving the Society as Secretary-Treasurer and Managing Editor for the last 37 years. If my service has been useful to the Society, it is due to the splendid and sympathetic cooperation of the national officers, the Councils, the Editors, the various national committees and to the cooperation of members and authors. To all I extend my sincere thanks. I also wish to express my thanks to Miss Helen McCabe and her two assistants for the very sterling and conscientious devotion to the Society during the many years, as assistant to the Secretary and Managing Editor.

It may not be out of order to cite the abbreviated resolution of the Council, prepared at their annual meeting in April 1950, on the occasion of my retirement from my professorship at the College of the City of New York, part of which resolution is given below:

"Whereas we individually and collectively desire to express to Prof. A. J. Goldforb our sincere thanks for his efficient and untiring efforts to the Society during the last 27 years . . .

"Resolved that the Council of the Society for Experimental Biology and Medicine, as an expression to him of its appreciation of his services, place this statement upon the official minutes of the Society."

I now turn over the multiple responsibilities to my successor, Dr. Walter S. Root.

# SUGGESTIONS TO AUTHORS

1. When manuscript is submitted it is assumed that *no other,* based on the same research, has been published previously, nor will be published simultaneously.

2. a. Manuscripts should be as *concise* as possible, yet *sufficiently* detailed to permit critical appraisal.
   b. Manuscripts should be *double* or *triple* spaced.

3. Manuscripts of *non-experimental* researches, or those with *inadequate controls* are not acceptable.

4. *Unnecessary subdivision* of a research into several manuscripts is not acceptable.

5. a. Manuscripts devoted to *improvement of procedure or of apparatus* may be accepted when a new principle is involved or when decidedly superior biological results are obtained. Evidence of such superiority should be given.
   b. *Confirmatory* or *negative* reports may be accepted only when they are of obvious biological significance.

6. Length of manuscripts averages 3 printed pages, including tables, charts, bibliography. Beyond this length, to 10 printed pages only by approval of Editors.

7. *Title* limited to 15 words.

8. *Conclusions* should be based upon the experimental data submitted.

9. a. *Illustrations.* Descriptive matter should not extend beyond the curves. Avoid large blank spaces.
   b. Explanations and numbers should be legible after reduction of Figure to one column.
   c. Drawings should be on white or blue tracing paper. Figures and drawings should be in black India ink, not typewritten.
   d. Half-tone Figures should be sharply contrasted, on white glossy paper.
   e. Illustrations larger than manuscript should be accompanied by smaller photograph.
   f. Illustrations should be grouped to fit printed page.

10. *Tables* should have concise heading, should be as simple as possible, and arranged preferably for vertical position for single or double column. For identical values use ditto marks, for similar phrases use "idem."

11. a. Only essential *citations* should be submitted, and arranged numerically at end of manuscript.
    b. Reduce number of citations by reference to recent reviews.
    c. "Personal communication" "unpublished," numerous abstracts should be excluded from bibliography. If manuscript has been accepted for publication, include it in bibliography, giving journal, year, etc. If *not accepted do not include it* in bibliography.

12. *Duplication* of table, graph and text is not acceptable.

13. *Trade* or *popular name* or *abbreviation* of a chemical may be used only when preceded by chemical or scientific name. Thereafter any of these names or abbreviations may be used. Trade name should begin with a capital letter.

14. *Structural formulae* of chemicals should be reduced to absolute minimum.

15. The Proceedings is copyrighted.

16. a. Authors are expected to discourage circulation of reprints for commercial purposes.
    b. *Reprints* are limited to 1300.

17. Changes in *galley proof* should be absolutely minimal. Excessive changes are charged. Galley proof of non-member's article is sent to sponsor.

18. *Sponsor* is held responsible for non-member's manuscript. Sponsor should write stating (a) how familiar he is with the author and his research, (b) whether author is scientifically reliable, (c) that manuscript conforms to "Suggestions to Authors," (d) that he has *critically* examined the manuscript, (e) wherein the manuscript is a significant contribution to science, and (f) that he assumes financial responsibility involved. Sponsor should be in the same laboratory or recent past laboratory as the author. Where not of the same or recent past laboratory, he should specify why such letter comes from a member at another laboratory.

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Notify the General Secretary at least one month *before change of address.* Editorial office is closed during August.